EFFECTS OF PH AND TRANSGLUTAMINASE ADDITION ON THE PROPERTIES OF FABA BEAN PROTEIN BASED FILMS

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submitted by **MELİSSA KOCATEPE** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Food Engineering, Middle East Technical University** by,

Prof. Dr. Naci Emre Altun
Dean, **Graduate School of Natural and Applied Sciences** _____

Prof. Dr. Hami Alpas
Head of the Department, **Food Engineering** _____

Prof. Dr. Serpil Şahin
Supervisor, **Food Engineering, METU** _____

Prof. Dr. Servet Gülüm Şumnu
Co-Supervisor, **Food Engineering, METU** _____

Examining Committee Members:

Prof. Dr. Halil Mecit Öztop
Food Engineering, METU _____

Prof. Dr. Serpil Şahin
Food Engineering, METU _____

Prof. Dr. Servet Gülüm Şumnu
Food Engineering, METU _____

Assoc. Prof. Dr. Leyla Nesrin Kahyaoğlu
Food Engineering, METU _____

Assist. Prof. Dr. Kübra Ertan
Food Engineering, Burdur Mehmet Akif Ersoy University _____

Date: 29.11.2024

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname : Melissa Kocatepe

Signature :

ABSTRACT

EFFECTS OF pH AND TRANSGLUTAMINASE ADDITION ON THE PROPERTIES OF FABA BEAN PROTEIN BASED FILMS

Kocatepe, Melissa
Master of Science, Food Engineering
Supervisor: Prof. Dr. Serpil Şahin
Co-Supervisor: Prof. Dr. Servet Gülüm Şumnu

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Biodegradable packaging is a growing trend as it is an eco-friendly alternative to synthetic polymers. Although natural polymers have certain limitations when used as packaging material, these limitations can be overcome by incorporating other functional components. In this study, films were made using the casting method. Faba bean protein concentrate was used as a base packaging material, and microbial transglutaminase (MTGase) was added to cross-link the protein chains and improve the barrier properties of the films.

In the first part of the study, films were prepared at two different pH levels (pH 6 and pH 8) and with varying dosages of MTGase (0, 5U, and 10U). Films made at pH 8 gave better results regarding WVP and mechanical properties due to the higher solubility of the proteins at pH 8. In addition, the degree of cross-linking with MTGase was higher at pH 8. The addition of MTGase at dosage of 10U improved the physical properties of the films.

In the second part of the study, thyme oil was added to film-forming solutions at pH 8. Although thyme oil addition improved antioxidant capacity and provided antimicrobial benefits, it affected the mechanical and barrier properties of the films

negatively. In addition, it was observed that thyme oil addition reduced the degree of cross-linking of the films. In order to achieve a successful active packaging film with high antioxidant capacity and antimicrobial activity, the addition of 1% thyme oil to faba bean protein film crosslinked with 10U MTGase is recommended.

Keywords: Biodegradable Film, Faba Bean Protein, Transglutaminase, Thyme Oil, Active Film

ÖZ

pH VE TRANSGLUTAMİNAZ İLAVESİNİN BAKLA PROTEİNİ BAZLI FİLMLERİN ÖZELLİKLERİ ÜZERİNE ETKİLERİ

Kocatepe, Melissa
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Prof. Dr. Serpil Şahin
Ortak Tez Yöneticisi: Prof. Dr. Servet Gülüm Şumnu

Kasım 2024, 111 sayfa

Biyobozunur ambalajlar, sentetik polimerlere çevre dostu bir alternatif olmalarından ötürü büyüyen bir trenddir. Doğal polimerlerin ambalaj malzemesi olarak kullanıldığında bazı dezavantajları olmasına rağmen, diğer fonksiyonel bileşenlerin eklenmesiyle bunların üstesinden gelinebilir. Bu çalışmada, temel paketleme malzemesi olarak bakla proteini konsantresi kullanılmış ve protein zincirlerini çapraz bağlamak ve filmlerin bariyer özelliklerini geliştirmek için mikrobiyal transglutaminaz eklenmiştir.

Çalışmanın ilk bölümünde dökme yöntemi kullanılarak iki farklı pH seviyesinde (pH 6 ve pH 8) ve değişen mikrobiyal transglutaminaz dozajlarında (0, 5 ve 10 birim) filmler yapılmıştır. Proteinlerin çözünürlüğünün daha yüksek olması nedeniyle pH 8'de yapılan filmler, su buharı geçirgenliği ve mekanik özellikler açısından daha iyi sonuçlar vermiştir. Ayrıca, transglutaminaz ile çapraz bağlanma seviyesinin pH 8'de daha yüksek olduğu görülmüştür. Transglutaminaz ilavesi 10 birim olduğunda filmlerin fiziksel özelliklerinde iyileşme olduğu görülmüştür.

Çalışmanın ikinci bölümünde ise pH 8'deki film çözeltilerine kekik yağı ilave edilmiştir. Kekik yağı ilavesi antioksidan kapasitesini arttırıp antimikrobiyal fayda sağlamasına rağmen, filmlerin mekanik ve bariyer özelliklerini olumsuz yönde etkilemiştir. Ayrıca kekik yağı ilavesinin filmlerin çapraz bağlanma derecesini de azalttığı gözlenmiştir. Yüksek antioksidan kapasiteye ve antimikrobiyal aktiviteye sahip başarılı bir aktif film elde etmek için, 10U MTGase ile çapraz bağlanmış bakla proteini filmine %1 kekik yağı eklenmesi önerilmektedir.

Anahtar Kelimeler: Biyobozunur Film, Bakla Proteini, Transglutaminaz, Kekik Yağı, Aktif Film

To my mother

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CHAPTER 1

INTRODUCTION

1.1 Food Packaging

In today’s fast-paced world, people's lifestyle choices are rapidly changing due to urbanization, changes in job types, and the influence of globalization. As more people move to urban areas, they often have busier lives since modern jobs frequently require long hours, and people start having less time to cook every meal at home. These changes collectively increase the demand for packaged foods, which meet the need for speed, variety, and accessibility. A recent market report published by Mordor Intelligence (2024) estimates the global market size of ready-to-eat meals as 402.90 Billion USD annually, which is expected to increase to 512.74 Billion USD by 2029. This growth underlines the importance of ongoing developments in packaging technologies and materials, as effective packaging not only preserves the quality and freshness of ready-to-eat meals but also enhances convenience and shelf-life, which meets the evolving preferences and lifestyle demands of modern consumers.

Packaging materials play a significant role when it comes to the quality of the product. They should provide robust protection against physical damage, microbial contamination, moisture loss, and oxidation to maintain food quality and safety throughout its shelf-life. Effective packaging extends freshness by controlling environmental factors like temperature, humidity and light exposure. Ideal materials possess excellent barrier properties against gases, moisture and light, which are crucial for preventing spoilage and preserving product integrity. In an overview published by Ibrahim et al. (2022), the most common packaging materials in the food

market are listed as plastics, glass, metal and paper, each having their own advantages and disadvantages. Plastics like PET and HDPE are lightweight, versatile, and offer excellent barrier properties against moisture and gases. However, they pose environmental concerns due to their non-biodegradable nature and potential for leaching harmful chemicals. Glass is inert, preserving taste and quality. However, it is heavy, brittle, and energy-intensive to produce and transport. Metal cans are durable, provide strong barrier protection, and are recyclable, yet they may alter taste and pose potential health risks from internal coatings. Paper and cardboard are renewable, biodegradable, and offer good printability for branding, but they provide limited barrier properties and can be susceptible to moisture and tearing.

1.2 Biodegradable Food Packaging

Mendes and Pedersen (2021) presented recent data in their paper, indicating that plastics currently constitute 26% of packaging materials, and this usage is projected to double within the next 20 years. However, their environmental drawbacks are increasingly hard to ignore. Espitia et al. (2014) note that less than 5% of all plastics are recycled. Since plastic materials are resistant to microbial degradation and need several hundred years to degrade in nature fully, the accumulation of 95% of the plastic material creates significant pollution and has a negative impact on the environment. In addition, the production of plastics heavily relies on fossil fuels and contributes to greenhouse gas emissions and climate change, which is a significant threat to wildlife and the ecosystem. Therefore, there is a growing need and shift towards biodegradable packaging materials derived from renewable resources, which offer a promising solution to the environmental challenges of traditional plastics.

Biodegradable packaging is a growing trend to promote sustainability, as the material used in biodegradable packaging can be more easily broken down in nature. Through the action of microorganisms like bacteria or fungi, these materials decompose and

return to nature without harming the environment. This can significantly reduce the ecological footprint of food packaging.

There is increasing attention on biodegradable polymers obtained from renewable sources, and many studies are being conducted on this subject every year. Polysaccharides such as starch and cellulose, animal and plant proteins, and lipids can be used in development studies, each having their own advantages and disadvantages. For example, films made with highly polar polymers like starch and protein may exhibit high water vapor permeability (WVP) due to the higher hygroscopicity of these polymers while having low gas permeability (Wihodo & Moraru, 2013). On the other hand, lipids act as a strong water barrier due to their hydrophobic characteristics (Muscat et al., 2013), may exhibit a plasticizing effect on the film and improve flexibility (McHugh, 2000). Biodegradable materials often cannot be readily used alone in packaging developments since their barrier properties and stability may not be sufficient to be used as food packaging materials (Rocha et al., 2018). Therefore, it is a common and effective practice to use these biodegradable materials in combination or add some modifications to biodegradable material in order to improve its protective characteristics.

There are different methods to produce biodegradable films. These methods diverge from each other by several factors, such as pore formation, thickness of the film, and involvement of organic solvents (Suntornnond et al., 2015). Depending on the specific need, a suitable production method should be selected. For instance, the freeze-drying method can be used when a porous film is needed. Spin casting is another method that can be used to obtain thin films (0.5–80 μm), but it involves organic solvents and needs a purification step. Water-based casting is one of the most popular methods, as it does not require expensive equipment, requires limited organic solvent usage, and is suitable for natural and hydrophilic biodegradable polymers. However, it has some drawbacks, such as relatively thicker (25–200 μm) film formation and low mechanical strength. On the other hand, these drawbacks can

be overcome by incorporating other methods, such as plasticizer addition or cross-linking methods.

Depending on the main ingredient, multiple methods can be used to improve biodegradable films. When starch is the main component of the film, acetylation is a common practice to provide mechanical strength and reduce hydrophilicity. Schmidt et al. (2019) studied the effects of acetylation levels on cassava starch and observed that acetylation indeed increased the tensile strength and reduced the solubility and WVP of the film, which helped to obtain a better packaging material. When protein is the primary source of the biodegradable film, cross-linking agents are a popular choice for achieving a more robust packaging material. They form a covalent or noncovalent bond between polymer chains and provide additional support. Azerado and Waldron (2016) reviewed some of the cross-linking agents, such as aldehydes and citric acid, and concluded that cross-linking agents may help to improve the packaging characteristics of the film, depending on the original nature of the primary polymer or processing conditions. For instance, amino groups used in cross-linking are favored by neutral or alkaline pH, and transglutaminase enzyme is highly temperature dependent. Therefore, raw materials and process conditions should be taken into consideration while selecting a suitable cross-linking agent.

1.2.1 Protein-based biodegradable films

Over the years, many researchers have spent their time on the development of protein-based films as an alternative to traditional packaging materials. Proteins provide a nutritious value to films, which is particularly important in edible films. In addition, due to the presence of nitrogen components, protein films can act as fertilizers while degrading in nature. Depending on the protein source, they may also bring mechanical or barrier advantages, which can be further improved with different methods or components. In general, proteins are combined with carbohydrates and lipids, where mechanical stability coming from proteins is supported by a gas barrier

properties of carbohydrates and hydrophobicity of lipids (Murrieta- Martínez et al., 2018).

Protein-based films can be developed by using either animal or vegetable-based sources. Casein, whey protein, and gelatin are some of the most popular animal-based proteins studied in film forming, whereas corn zein, wheat gluten, and soy proteins are trending plant-based proteins in this area (Mihalca et al., 2021). Amino acid composition and arrangement affect the chemical potential of the protein since charged, polar, and non-polar amino acids act differently. Based on the distribution of the amino acids along the protein chain, electrostatic interactions, hydrogen bonding, van der Waals forces, covalent bonding, and disulfide bridges may occur. These bonds help the formation of the protein matrix and influence the film properties (Dangaran et al., 2009).

Proteins can be classified by their sedimentation speed. Svedberg (S) unit is used to denote the sedimentation speed of a particular molecule. The most common storage proteins are 2S (such as albumins), 7S (vicilins), 11S (legumins), and 15S (such as glutenin) proteins, each having their own functionalities. Increasing numbers before the Svedberg unit implies that the molecular weight is rising; therefore, the molecule has a higher sedimentation speed. Increasing molecular weight results in an improvement in viscoelasticity and an increase in film-forming ability. Due to their smaller molecular size, 2S proteins are highly soluble and cannot form films when used alone (Shewry et al., 1995). 7S protein films are usually brittle, but they can aid in incorporating lipids due to their high emulsification capacity (Kinsella, 1979). 11S proteins have great gelation and emulsifying properties, which makes them highly suitable for film formation. Films made with 11S proteins are strong and flexible, with good gas and water barrier properties (Barac et al., 2010). Finally, 15S proteins have unique viscoelastic properties and can form highly cohesive and elastic films (Day et al., 2006).

Protein-based films can be formed using the casting method. Proteins must be dissolved in a suitable solvent to prepare a film-forming solution to achieve proper

films. After adding other functional ingredients, such as plasticizers or cross-linking agents, it is essential to homogenize the film-forming solution in order to achieve an even distribution of components and emulsification of lipids, which results in an improvement in film characteristics. Additionally, solutions are generally degassed by vacuum or ultrasonication to remove air pockets inside the film-forming solution. When these air pockets are not removed, they may negatively affect the mechanical or barrier properties of the film, which may lead to film disruption. After appropriate preparation, the film-forming solution is spread onto a surface, and the solvent is evaporated (Gomez-Estaca et al., 2016). In order to form a film, the pH of the film-forming solution should be adjusted. In their article, Shiku et al. (2003) explained that the pH of the solution should not be close to the isoelectric point of the protein, as proteins tend to coagulate when the pH is too close to the isoelectric point, which results in the inhibition of film formation. Similarly, the pH of the solution should not be extremely acidic or alkaline since strong charges along the protein chain create repulsive forces between amino acids, which block the formation of films (Mauri & Anon, 2006).

Another critical step in producing protein-based films is the denaturation of the protein to unfold the globular structure and allow the formation of new interactions. When all amino acids are exposed through denaturation, inter- and intramolecular interactions are reinforced, resulting in a stronger film with better barrier properties (Guerrero et al., 2010). This denaturation can be achieved with several methods, such as heat treatment, pH change, or pressure. Combining different denaturation methods helps to ensure complete denaturation and, therefore, better film formation.

Over the years, numerous studies have investigated protein-based films using both plant- and animal-derived protein sources. Among plant-based sources, soy and pea proteins are particularly popular. For example, Kowalczyk et al. (2011) studied the effects of pH, plasticizer content, and processing conditions on pea protein isolate films. Similarly, Kang et al. (2024) tested the impact of transglutaminase enzyme on soy protein films under different pH conditions. In terms of animal-derived proteins,

whey protein and gelatin are widely studied. McHugh et al. (1994), for instance, evaluated the barrier properties of plasticized whey protein films after exposure to different heat treatment and pH conditions. Likewise, Liu et al. (2017) studied the effects of glycerol content and transglutaminase treatment on gelatin-based films. Research also includes studies combining animal and plant proteins. Weng and Zheng (2015) investigated the influence of transglutaminase on tilapia scale gelatin films incorporated with soy protein isolate. Ahammed et al. (2021) tested different zein-gelatin ratios in film formation and analyzed the effects of transglutaminase on film properties. Finally, combinations of proteins and carbohydrates in film formation are also widely studied. For example, Carvalho et al. (2020) examined the impact of thyme oil on whey protein isolate-cellulose nanofiber composite films. Likewise, Rosseto et al. (2021) researched the effects of transglutaminase treatment and aging on starch-gelatin films.

1.2.2 Active biodegradable films

Active packaging films are obtained when bioactive compounds such as antioxidants, antimicrobials, vitamins, and flavorings are incorporated into the matrix. These films may interact with the food material to provide additional food safety or enhance sensory characteristics. For example, Rivero et al. (2013) tested the antimicrobial effectiveness of propionic acid incorporated chitosan active films in pastry dough and observed the inhibitory impact on *S.aureus (ATCC 25923), Salmonella* spp. *Candida* spp. and *Penicillium* spp. In another study, Perez et al. (2011) observed the inhibition of the growth of several *E.coli* strains when potassium sorbate-added whey protein-based films were used. Furthermore, the enrichment of biodegradable films with essential oils comes with multiple benefits: it brings an active packaging attribute to the film and may alter the film's physicochemical structure to improve barrier properties. The incorporation of oregano essential oil in cassava starch-chitosan film decreased the WVP of the films while inhibiting the growth of *Bacillus cereus* ATCC 25923, *E. coli* ATCC 25922, *S. Aureus* FRI196e,

and *S. enteritidis* (Pelessari et al., 2009). Rambabu et al. (2019) tried incorporating different dosages of mango leaf extract in chitosan-based films and achieved significantly lower peroxide levels in cashew nuts compared to control samples. When cashew nuts were only wrapped inside chitosan-based films, the peroxide level at the end of 28 days was measured as 6.34 mEq/kg, while in the case of 5% mango leaf extract added chitosan film, it was2.88 mEq/kg. On the other hand, film composition, stability, and structural properties are essential factors in achieving a compelling, active film. Oussalah et al. (2004) tested pimento and oregano essential oils in milk protein-based biodegradable films and noticed that the release rate of phenolic components and the moisture content of the film are essential criteria for achieving the desired antioxidant and antimicrobial benefits. Therefore, active films may need structural modifications in order to provide maximum benefits.

1.3 Film Materials

1.3.1 Faba bean protein concentrate

Faba bean (*Vicia faba* L.) is a type of legume that can be used as a biodegradable film-forming material. It has a high nutritional value, is easy to grow in various climate conditions, and enriches the soil while decomposing due to the presence of nitrogen components (Karkanis et al., 2018). The protein content of faba bean can be up to 35%, where the ratios of 2S proteins are 17%, 7S proteins are 33%, 11S proteins are 45%, and 15S proteins are 5% (Boye et al., 2010), which makes faba bean a good option for biodegradable films. In a 15% (w/w) aqueous solution, it exhibits a denaturation temperature with a T_{onset} of approximately 89 ºC and a T_{peak} of about 94°C, both of which decrease as the pH moves farther from the isoelectric point (Badjona et al., 2024).

Different grades of faba bean can be found on the food market, ranging from faba bean flour to faba bean protein concentrates and, finally, faba bean protein isolates. Faba bean flour is typically produced by dehulling, followed by grinding. It usually

has a nutritional composition similar to that of the faba bean itself. To produce faba bean protein concentrates, faba bean flour is processed further by air classification, which separates the fine protein particles from the coarse starch particles. By doing this, the protein ratio of the concentrate can be increased up to 65%. For protein isolates, a wet extraction process is needed. Firstly, proteins must be dissolved in a solvent like water by adjusting the pH. Then, dissolved protein is precipitated by bringing the pH to the isoelectric point, centrifuged for separation, and lastly, dried (Sosulsky & McCurdy, 1987). Nutritional compositions of faba bean flours, concentrates, and isolates are summarized in Table 1.1.

Table 1.1. Mean compositions of faba bean flour, concentrate, and isolate (%)

Component	Faba Bean Flour	Faba Bean Protein Concentrate	Faba Bean Protein Isolate
Protein	29.2	63.3	86.3
Fat	1.4	2.9	2.0
Fiber	1.6	2.9	0.6
Starch	51.2	3.7	1.8
Ash	2.6	5.4	3.9

Several studies have been conducted with faba bean fractions for functional purposes. In a study by Vogelsang-O'Dwyer et al. (2020), the functionality of faba bean protein concentrate and isolate was compared in various criteria. It was observed that faba bean concentrate outperformed isolate in protein solubility, foaming capacity, and gel strength; however, isolate gave better foam stability and digestibility results. Koberstein-Hajda and Dickinson (1996) found that faba bean protein fractions, which are acetylated, 7S enriched, and 11S enriched, have the potential to replace Span 80 as the primary lipophilic emulsifier in water-in-oil-in-water (W/O/W) double emulsions. Jiang et al. (2020) tested the performances of faba bean flour in emulsion gels made with whole faba bean flour and alpha-amylase

hydrolyzed faba bean flour. While the former flour contains all macro components, the latter contains no starches. As a result, it was observed that starch hydrolysis improved the emulsion stability and gel strength. These improvements were mainly attributed to high viscosity coming from starch and interruption of protein gel network formation. Lastly, Hu et al. (2023) tried using concentrated emulsion gels made with faba bean isolate in 3D food printing. Different dosages of faba bean isolate, different pH conditions, and salt addition were tested, and all gels were passed through heat treatment at 90 ºC for 30 min. Eventually, it was concluded that faba bean isolate gels could be effectively optimized and used in 3D printing as they exhibit high gel strength, provide emulsion stability, and have good heat resistance.

Contrary to gel formation and emulsion stability, the usage of faba bean protein in biodegradable films has not been widely investigated. Saremnezhad et al. (2011) evaluated faba bean proteins extracted from faba bean flour in film formation. In order to isolate proteins, the alkaline extraction method was used, and the proteins obtained were tested in pH values of 7, 9, and 12 with different glycerol concentrations (40%, 50%, and 60% w/w of the protein isolates). It was observed that pH and glycerol content affect WVP, mechanical properties, color, and solubility of the films. However, the effects of pH change were much more significant than glycerol content, except for % elongation at break. In general, pH increase resulted in an improvement in film properties and increased solubility. A similar study was conducted by Montalvo-Paquini et al. (2014), where isolated faba bean proteins were tested at pH 7, 8.5, and 10 with a glycerol concentration of 50% (w/w) of the protein isolate. The results were parallel to those of the previous study; increasing the pH improved the WVP, tensile strength, and % elongation at the break of the films while increasing the solubility simultaneously. Rojas-Lema et al. (2021) incorporated varying dosages (1%, 3%, 5%, and 7% w/w of faba bean protein) of cellulose nanocrystals (CNC), which were obtained from pine cone into faba bean protein solution to strengthen film forming properties. As a result, tensile strength, Young's modulus, and thermal stability were increased, while % elongation at break, moisture content, water solubility, water vapor and oxygen transfer rates decreased. It can be

concluded that the addition of CNC improved the film-forming properties of faba bean-based films. In the subsequent article (Rojas-Lema et al., 2023), different dosages of lignin (2.5%, 5%, 7.5%, and 10 % w/w of faba bean protein), which was also extracted from a pine cone, were tried in faba bean protein-based films. Similarly, adding lignin increased tensile strength and thermal stability while decreasing moisture content and water vapor transfer rate. Meanwhile, lignin acted as a plasticizer in faba bean-based films, and % elongation at break significantly increased at 5% lignin addition. However, as lignin dosage increased to 7.5% and 10%, pores formed, and microcracks occurred. Yildiz et al. (2022) compared the performances of glutaraldehyde and citric acid cross-linked faba bean flour-chitosan-curcumin-based films, as glutaraldehyde is considered to be hazardous. Although citric acid and glutaraldehyde bring different attributes to the films, citric acid can replace glutaraldehyde in faba bean-chitosan-curcumin films as a cross-linking agent, especially when water sensitivity is an important criterion. Briefly, citric acid created more elastic films, while films made with glutaraldehyde were more brittle. Lastly, the antioxidant and antimicrobial effects of sumac addition on faba bean flour-based films were examined by Emir et al. (2023). Sumac addition successfully suppresses the growth of *S.aureus* (ATCC 43300) at 37 °C and doubles the shelf life of chicken breast under refrigeration (4 ºC) at higher dosages. However, it was not enough to create protection against *E.coli* (ATCC 11229). Moreover, sumac addition significantly changed the physicochemical properties of the films by decreasing the moisture content and WVP while increasing the solubility, tensile strength, and % elongation at break.

1.3.2 Glycerol

Glycerol, also known as glycerine, is a colorless, odorless, and sweet-tasting polyol widely used in the food industry. It acts as a humectant, which also provides plasticizing and lubricating properties. While developing biodegradable films, glycerol serves as a plasticizer to provide flexibility and reduce the brittleness of

biodegradable films. This is achieved by interrupting polymers' intra- and intermolecular hydrogen bonding, where glycerol fills the space between polymer chains and promotes polymer-glycerol interaction. There are four different theories explaining the plasticizing effect of glycerol, summarized by Ben et al. (2022) as:

1. Lubricity theory: Plasticizers act as a lubricant, and their presence reduces the friction between crystalline polymer chains. Therefore, polymer chains easily slide over each other, which brings flexibility to the film.

2. Gel theory: There are several points along the polymer chains, where hydrogen attraction occurs. Glycerol shields the attachment points, reducing the interaction between molecules and providing mobility to polymer chains.

3. Mechanistic theory (solvation-desolvation equilibrium): An addition to gel theory, where glycerol molecules are also not stationary, and their movement also disrupts the polymer matrix and brings additional plasticity.

4. Free volume theory: Glycerol molecules take up space within the polymeric network, and the polymers cannot return to initial rigidity after swelling. Thus, this increased free volume provides higher mobility.

Although glycerol is improving the plasticity of biodegradable films, higher dosages may have a negative impact on other film properties. In the study of Hidayati et al. (2021), it was found that increasing dosages of glycerol increases water solubility, which improves biodegradability but disrupts the protective behavior of the film. Farahnaky et al. (2012) showed that higher amounts of glycerol also cause an increase in WVP due to increased hygroscopicity from glycerol. Therefore, the glycerol amount should be optimized in order to achieve the desired balance between film properties.

Similar studies have also been conducted in protein-based films, and the results are in parallel. Kokoszka et al. (2010) found that increasing glycerol percentages in the same amount of soy protein solutions increases WVP. In a study by Hopkins et al.

(2019), the effects of glycerol percentages (50%, 75%, and 100% w/w of protein) were observed in different plant-based protein films. Higher glycerol levels increased the film thickness and WVP, and reduced the tensile strength for all protein sources. Additionally, higher glycerol levels increased the % elongation at break and opacity in faba bean-based films. When all studies are considered, it can be concluded that glycerol content can be adjusted depending on specific needs.

1.3.3 Microbial transglutaminase enzyme (MTGase)

As Kashiwagi et al. (2002) briefly explained, transglutaminase (TGase, protein-glutamine γ-glutamyltransferase) is a transferase enzyme that catalyzes the formation of an isopeptide bond between γ-carboxamide groups of glutamine amino acid and the ε-amine groups of other compounds, mainly proteins (acyl-transfer reaction). When lysine amino acid is the acyl receiver, this transfer reaction induces the cross-linking process, which forms ε-(γ-Glu)Lys. If free amine groups are absent, water acts as an acyl acceptor, and TGase catalyzes the deamidation reaction. These reactions catalyzed by TGase cause significant changes in the physical and chemical properties of proteins, such as changes in viscosity, thermal stability, elasticity, and resilience. TGase-induced reactions are summarized in Figure 1.1.

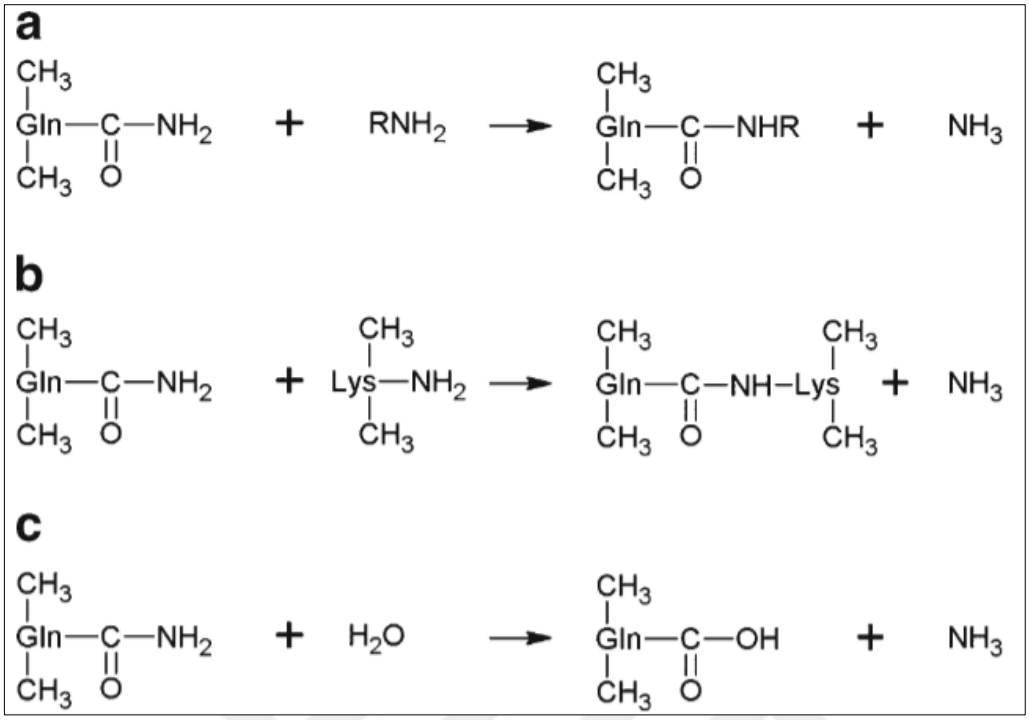


Figure 1.1. TGase induced reactions a)acyl-transfer reaction; b) cross-linking reaction between Gln and Lys residues of proteins or peptides; c) deamidation (Kieliszek & Misiewicz, 2013)

TGase can be derived from various sources, such as animal tissues (liver and blood), plants, and microorganisms. Due to complex and expensive extraction processes, animal and plant-based TGase has certain limitations. On the other hand, microbial transglutaminase (MTGase) is produced via fermentation processes of specific microorganisms, such as *Streptomyces mobaraensi* (Zhang et al., 2009). As microbial sources are more prevalent than animal or plant sources, MTGase has a broader usage in the industry. In addition, since the production of MTGase takes place in a more controlled environment, end product specifications are more easily maintained, and the product quality is standardized.

Several studies have been conducted on the effects of TGase on proteins. Shand et al. (2006) studied the effects of MTGase dosage, incubation time, and incubation temperature on pea protein gels and compared these with soy protein gels. As a

result, it was observed that MTGase treatment on pea protein gels improved strength and elasticity and helped achieve a gel similar to soy protein gels. Findings show that the same gel strength and elasticity can be achieved by adjusting the enzyme dosage and incubation conditions. For example, similar gels were achieved by treating soy protein isolate with 0.35% (w/w) MTGase, incubating at 50º C for 45 minutes, and treating with 0.6% (w/w) MTGase and incubating at 4º C for 7 hours. In another study, Schlangen et al. (2023) compared the effects of TGase dosage and incubation temperatures on pea and mung bean protein gels. In this study, proteins were not denatured before enzyme treatment, and it was observed that the structure of protein chains and amino acid accessibility significantly affected the gel characteristics. Therefore, it was concluded that TGase effects vary depending on the protein source. Lastly, Nivala et al. (2021) tested the effects of heat and TGase treatment on the gelling and emulsifying properties of faba bean protein. The results showed that while there were no significant differences between samples regarding emulsion capacity and stability, gelling properties and water-holding capacity increased with heat and enzyme treatment.

Due to the functional changes occurring during MTGase treatment, the enzyme is widely used in the food industry, especially in the meat and dairy industries. As cross-linking helps to create additional chemical bonds between protein chains, MTGase can bind small meat pieces together and create a single, large piece of meat. Moreover, additional bonds harden the protein gels, which is also helpful in surimi production. In yogurt production, MTGase helps to eliminate serum separation caused by lactic acid fermentation since MTGase increases the water-holding capacity of milk gel (Yokoyama et al., 2004). Recently, MTGase found its place in the plant-based food industry as well. In tofu production, it is used as gelling support to create more rigid and more durable gels. In noodles, it can prevent the deterioration occurring after cooking. Lastly, it can improve the texture and color of veggie burgers (Forghani et al., 2017).

The effects of MTGase usage in film formation have also been studied frequently. Tang et al. (2005) evaluated the effects of MTGase on cast films prepared with soy protein isolate and found that some barrier properties, such as tensile strength and surface hydrophobicity, were increasing with MTGase treatment, while WVP remained the same and % elongation at break, moisture content, and solubility were decreasing. In this study, different plasticizer combinations were also tried and it was found that both glycerol and sorbitol have their own advantages in different criteria. Therefore, the most suitable plasticizer should be decided depending on the needs. In the subsequent article, Jiang et al. (2007) tried different dosages of MTGase on soy protein-based cast films and noticed that the effects of MTGase vary significantly with enzyme concentration, pH of the solution, and drying conditions. In addition, these parameters need to be optimized to inhibit the formation of protein aggregates, which may cause deformations in the films and affect mechanical properties. They suggest a low enzyme concentration, alkaline pH range, and air-drying temperature to avoid aggregation. In another study in 2010, one of the factors studied by Patzsch et al. was the MTGase reaction conditions. In this study, they evaluated different enzyme treatment conditions in sodium caseinate films, which were MTGase addition at 50°C and film drying at room temperature, MTGase addition and film drying at 50°C, and lastly, MTGase treatment at 50°C for 15 min, inactivation for 10 min at 90°C and film drying at room temperature. The results showed that the inactivation of the enzyme gave the lowest tensile strength, which was almost 40% lower than the others. Moreover, it was noticed that the presence of glycerol increased the activity of MTGase. Lastly, Tang et al. (2011) tested different dosages of TGase on red bean protein-based cast films and concluded that increasing dosages of TGase improves mechanical properties up to 10 U/g protein, but starts impairing the film after 20 U/g protein. In addition, they observed aggregations of proteins if the enzyme treatment was done poorly. Therefore, it is crucial to adjust the enzyme dosage, treatment, and drying conditions in order to achieve proper film formation.

1.3.4 Thyme oil

Essential oils are aromatic oily liquids obtained from different plant materials, such as oregano, thyme, and rosemary. Due to the inclusion of biologically active compounds, essential oils are considered antibacterial, antifungal, antiviral, insecticidal, and antioxidant bio-agents. Thyme oil is one of the most endorsed essential oils used in the food industry, providing antioxidant and antimicrobial benefits to the food system. It can be obtained from various plants, such as *Thymus vulgaris* and *Thymus zygis*, and it contains different phenolic components like thymol and carvacrol (Aljabeili et al., 2018). Typical composition of thyme oil obtained from *Thymus vulgaris* (Shabnum & Wagay, 2011) is summarized in Table 1.2. the major phenolic compound in thyme oil is thymol, and its molecular structure is shown in Figure 1.2. (Dong et al., 2020).

Table 1.2 Typical composition of thyme oil obtained from Thymus vulgaris

Components	Concentrations (%)	Components	Concentrations (%)
ά- thujene	2.84	γ-terpinene	14.08
ά- pinene	2.97	linalool	3.99
B- pinene	0.71	thymol methyl ether	1.78
mycrene	3.45	thymol	46.21
ά – terpinene	2.69	carvacrol	2.44
p-cymene	9.91	terpinyl acetate	0.68
limonene	1.23	β- caryophyllene	1.64
1,8-cineole	1.96	Other components	3.42

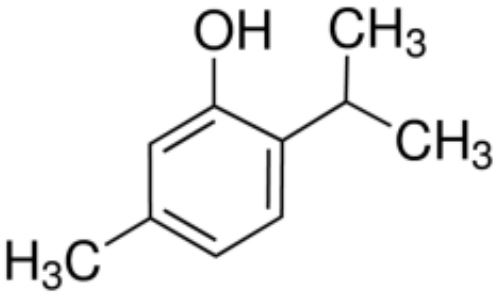


Figure 1.2. Chemical structure of thymol

Phenolic components present in thyme oil have hydroxyl groups, which can donate hydrogen atoms to free radicals. By doing this, they can neutralize free radicals and stop the chain reactions leading to lipid oxidation. When phenolic compounds donate hydrogen atoms, they become new radicals; however, they do not cause any oxidation since they are more stable and less reactive than free radicals (Youdim et al., 2002). Thymol and carvacrol are lipophilic components that can integrate into the lipid bilayers of microbial cell membranes. This integration disrupts the membrane structure, increasing its permeability, and eventually causes the leakage of vital cellular contents like ions and ATP. A disrupted cell membrane cannot maintain its metabolic processes anymore, which results in cell death (He et al., 2022).

Incorporating essential oils into packaging films is also a widely investigated topic. Numerous research has been conducted on thyme oil-added active biodegradable films and their antioxidant and antimicrobial effects. Hosseini et al. (2009) compared the antimicrobial effects of varying dosages of thyme, clove, and cinnamon essential oils in chitosan-based films. They found that thyme oil had the highest antimicrobial activity compared to other essential oils. In addition, it was observed that adding essential oil altered the mechanical and barrier properties of the films. Pires et al. (2011) studied the antioxidant capacity of thyme oil in hake protein-based films and found that increasing dosages of thyme oil increased the DPPH□ radical scavenging activity. 0.25 (mL/g protein) thyme oil addition resulted in 40% radical scavenging,

whereas the control sample without thyme oil only provided 15%. In addition, higher thyme oil levels resulted in lower tensile strength, % elongation at break, moisture content, and WVP. On the other hand, Jouki et al. (2014) found that increasing thyme oil concentrations up to 2% (v/v) increased WVP and % elongation at break in quince seed mucilage films. Similarly, Rahimi et al. (2023) noticed that increasing thyme oil dosage to 3.75% increases the WVP while reducing water solubility in gelatin-based films. To summarize, the physicochemical effects of thyme oil addition in biodegradable films vary significantly, depending on the biomaterial, dosage, and process. Both process conditions and thyme oil concentration need to be optimized in order to achieve the desired physicochemical characteristics.

1.4 Objectives of the Study

Faba bean is a versatile crop that can be easily grown in various climate conditions and serves as a sustainable plant-based protein source. Additionally, it contains high ratio of globulin proteins, making it an ideal choice for biodegradable film production. Similarly, MTGase enzyme has a good potential to be used in biodegradable film production as it can enhance structural integrity of the films and contribute to producing stronger films with improved barrier properties. In the literature, the number of studies conducted with faba bean protein as the main component of biodegradable films is limited. In addition, no study has been done on the effects of MTGase on faba bean protein-based biodegradable films. Therefore, the combined effects of faba bean protein and MTGase enzyme are unknown and need exploration.

This study aims to formulate and produce biodegradable films using faba bean protein concentrate, with varying dosages (0, 5U, and 10U) of MTGase enzyme. The study seeks to understand the film-forming behavior of this protein, the effects of crosslinking using MTGase, and how these components can be effectively used in creating sustainable packaging materials.

This study consists of two stages. In the first stage, the focus was to explore the influence of different pH levels on the properties of the biodegradable films. Therefore, films are produced at pH 6 and pH 8, and evaluated in terms of mechanical properties, thermal stability, barrier properties, and microstructure.

In the second stage, thyme oil was added to the films at selected pH to produce active packaging films. Structural changes, antioxidant capacity, and antimicrobial activity were compared in films with and without thyme oil.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The faba bean protein concentrate (Vitessence® Prista P 360) was supplied from Ingredion GmbH (Hamburg, Germany). The product was produced by dehulling, grinding, and air classification of *Vicia faba* beans, followed by a high-temperature short-time heat treatment to remove volatiles to neutralize taste (Ingredion, 2023). The typical composition of the product and amino acid profile provided by the supplier are summarized in Tables 2.1. and 2.2., respectively.

Table 2.1. Typical nutritional composition (%) of Vitessence® Prista P 360

Component	Vitessence® Prista P 360
Protein	59.5
Fat	3.7
Saturated Fat	0.7
Fiber	13.4
Carbohydrates	12.5
Sugar	1.7
Ash	5.7

Table 2.2. Typical hydrolyzable amino acid amounts (g) in 100 g of product

Amino acid	Typical amount	Amino acid	Typical amount
Histidine	1.2	Valine	2.7
Isoleucine	2.6	Glycine	2.4
Leucine	4.5	Arginine	5.6
Lysine	3.8	Proline	2.5
Methionine	0.4	Aspartic acid	6.3
Cysteine	0.6	Glutamic acid	9.6
Phenylalanine	2.5	Alanine	2.4
Tyrosine	1.8	Serine	2.8
Threonine	2.0	Hydroxyproline	N/A
Tryptophan	0.6		

The MTGase enzyme used in this study (Vertera® ProBite) was supplied from Novozymes A/S (Bagsværd, Denmark). The enzyme is produced by the bacteria *Bacillus licheniformis*, has 300 U/g activity (Novozymes, 2023), and is in liquid form. The enzyme is active at temperatures up to 70°C with an optimum range between 45-50°C and at a pH range of 4.5 to 8.5 with an optimum pH between 5.5-7.5. The suggested dosage is 0.025 g of enzyme per gram of protein, corresponding to 7.5U enzyme activity per gram of protein.

Thyme oil was purchased from Arı Mühendislik (Ankara, Turkey). Glycerol, hydrochloric acid (6.0 M), sodium hydroxide, tryptic soy agar, tryptic soy broth, glycine, and gallic acid were procured from Merck (Darmstadt, Germany). TNBS (2,4,6-trinitrobenzene sulfonic acid) solution (5% w/v in methanol) was ordered from Thermo Fischer Scientific (Waltham, MA, USA). Folin–Ciocalteu's phenol reagent (2.0 M), ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), methanol, potassium persulfate,

sodium carbonate and sodium hydrogen carbonate were obtained from Sigma-Aldrich (Darmstadt, Germany).

2.2 Film Preparation

In this study, the method developed by Ertan et al. (2024) was used to prepare film-forming solutions. Preliminary experiments were performed to identify the ideal dry matter and glycerol contents of the film-forming solutions, pH values and enzyme dosages to be compared, the ideal duration of ultrasound treatment, and the enzyme application procedure. Drying conditions were also standardized.

Film-forming solutions were prepared by dissolving 8% (w/v) faba bean protein concentrate in 100 mL distilled water. Solutions were stirred by a magnetic stirrer (SMHS-3-Hotplate Stirrer, Daihan Scientific Co, Daejeon, Korea) at 750 rpm for 30 min to ensure complete dissolution. Then, the pH of the solutions was adjusted by using a pH meter (SG2 SevenGo^{TM}, Mettler Toledo, OH, USA) to either 6 (with 1N HCl solution) or 8 (with 1N NaOH solution). Following pH adjustment, solutions were stirred for an additional 30 min at 750 rpm. Subsequently, solutions were placed into a water bath (WiseBath, Witeg Labortechnik GmbH, Wertheim, Germany) at 90ºC and rested for 30 min. After proteins were denatured, solutions were cooled down to 45-50ºC at room temperature. While stirring at 600 rpm with a magnetic stirrer, 2.5% glycerol (w/v) was added to the solutions, and stirring was maintained for an additional 30 min. Then, 1% thyme oil was added to the selected solutions at this stage. Later, all solutions were homogenized at 8000 rpm for 2 min with a high-speed homogenizer (IKA T18 Digital Ultra Turrax, Staufen, Germany). In order to eliminate the air bubbles of the solutions and achieve uniform films, solutions were degassed for 10 min with an ultrasonic water bath (OMNI 400, OMNI International the Homogenizer Company, USA) operating at 160 W and 20 kHz. Lastly, 0.72 g (5U) or 1.44 g (10U) of 10% (v/v) microbial transglutaminase solution was dosed into selected film-forming solutions and mildly stirred with a spoon while avoiding the formation of air bubbles.

In the following stage, 12.5 grams of film-forming solutions were poured into 90 mm LDPE petri plates, which were immediately put into a hot air oven (Binder ED 115, Tuttlingen, Germany) to be dried at 40ºC for 16 hours. After 16 hours, plates were removed from the oven and conditioned in a climatic chamber (TK 600, NÜVE, Ankara, Turkey) for 48 h at 20ºC and 50% relative humidity. Dried films were peeled off with a tweezer before the analysis.

Films were named based on the pH of the solution, enzyme activity, and presence of thyme oil. For example, FB_8_E10_T denotes the film with pH=8, 10U enzyme concentration, and with the addition of thyme oil (Table 2.3).

Table 2.3. Nomenclature of the films

Nomenclature	pH	Enzyme activity	Thyme oil % (v/v)
FB_6	6	0	0
FB_6_E5	6	5U	0
FB_6_E10	6	10U	0
FB_8	8	0	0
FB_8_E5	8	5U	0
FB_8_E10	8	10U	0
FB_8_T	8	0	1
FB_8_E5_T	8	5U	1
FB_8_E10_T	8	10U	1

2.3 Characterization of the Films

2.3.1 Thickness of the films

The thickness of the films was measured by a digital micrometer (LOYKA 5202, Loyka, Ankara, Turkey) with a precision of ±0.001 mm. Measurements were performed in three parallels and three repetitions for each sample. Data were collected from ten random locations of each film.

2.3.2 Moisture content and solubility of the films

In order to measure the moisture content (MC) and water solubility (WS) of the films, gravimetric analysis was used. Firstly, samples were cut into 2 cm×2 cm squares, and the initial weights (W_i) were recorded. After drying in the 105°C oven for 24 hours, dried films (W_d) were weighed again. MC (%) of the films were calculated by Equation (1):

$$\text{MC}\ (\%) = \frac{W_i - W_d}{W_i} x100 \qquad \text{Equation (1)}$$

Later, dried film samples were soaked into 30 mL distilled water and stored at room temperature for 24 hours. Then, samples were dried again at 105°C for 24 hours, and final weights (W_f) were noted. The solubility of the films was calculated by Equation (2):

$$\text{WS}\ (\%) = \frac{W_d - W_f}{W_d} x100 \qquad \text{Equation (2)}$$

Measurements were performed in three parallels and three repetitions for each sample.

2.3.3 Water vapor permeability of the films

The water vapor permeability (WVP) of the films was measured by following the ASTM E96/E96M-24 (ASTM International, 2024) standard. In the analysis, cylindrical permeability cups with a 2 cm diameter opening were filled with 30 mL of distilled water and covered with film samples. After tightening the screws, the cups were weighed and placed in a desiccator. Relative humidity inside the desiccator was kept low throughout the analysis using dried silica gel. Weight loss of cylindrical cups, relative humidity inside the desiccator, and temperature were measured every 60 min for 10 hours. The water vapor transmission rate (WVTR) ($g.h^{-1}.mm^{-2}$) was calculated by using Equation (3):

$$\text{WVTR} = -\frac{\text{slope}}{area} \qquad \text{Equation (3)}$$

where slope is the slope of the weight of the cups versus time graph ($g.h^{-1}$), and area is the area of the film covering the opening (mm^2). The water vapor permeability (WVP) ($g.h^{-1}.mm^{-2}.Pa^{-1}$) was calculated by using Equation (4) (Jia et al., 2009):

$$\text{WVP} = \frac{WVTR\ x\ \Delta x}{P_1 - P_2} \qquad \text{Equation (4)}$$

where Δx is the thickness of the film (mm), and P_1 and P_2 are the partial water vapor pressures inside the cup and the desiccator, respectively.

Measurements were performed in triplicates for each sample.

2.3.4 Color and opacity of the films

The colors of the film samples were measured by spectrophotometer (CR-410, Konica Minolta Inc., Tokyo, Japan), and parameters were denoted in CIELab color space (L*, a*, b*). The L* value shows the lightness, ranging between 0 (black) and 100 (white); the a* value shows the redness or greenness, ranging between +60 (red) and -60 (green); and the b* value represents the yellowness or blueness, ranging

between +60 (yellow) and -60 (blue). White calibration plate (L*=97.53, a*=-0.15, b*=1.58) was used as a reference, and total color difference (ΔE*) was calculated by using Equation (5) (Hill et al., 1997):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Equation (5)}$$

Whiteness index (WI) was calculated by using Equation (6) (Hirschler, 2012):

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2} \qquad \text{Equation (6)}$$

Yellowness index (YI) was calculated by using Equation (7) (Hirschler, 2012):

$$YI = \frac{142.86 b^*}{L^*} \qquad \text{Equation (7)}$$

For opacity measurements, the absorbance of the films at 600 nm (A600) was recorded, and the opacity of the films was calculated by using Equation (8) (Zhao et al., 2022):

$$\text{Opacity} = \frac{A600}{\Delta x} \qquad \text{Equation (8)}$$

where Δx is the thickness of the films in mm.

Measurements were performed in three parallels and three repetitions for each sample. Data were collected from three random locations of each film.

2.3.5 Mechanical properties of the films

Tensile strength (TS), % elongation at break (%EAB), and Young's modulus (YM) were calculated by using the data collected with a texture analyzer (CT3, AMETEK Brookfield, Middleboro, MA, USA). ASTM D882-18 (ASTM International, 2018) standard procedure was used during measurements. The films were cut into 80 x 20 mm rectangular strips. The trigger load was adjusted to 0.1 N; the test speed was set

as 0.40 mm.s^{-1}, and TA-DGA (Dual Grip Assembly) was used as a fixture. Tensile stress (MPa) was calculated by using Equation (9):

$$\text{Tensile stress} = \frac{F}{W\ x\ \Delta x} \qquad \text{Equation (9)}$$

where F (N) is the load applied to strips, W (mm) is the width of the film, which was 20 mm for all strips, and Δx (mm) is the thickness of the film. The maximum load achieved at the breaking point was recorded as TS.

Tensile strain was calculated by using Equation (10):

$$\text{Tensile strain} = \frac{L - L_i}{L_i} \qquad \text{Equation (10)}$$

where L_i (mm) and L (mm) are the initial and achieved lengths of the films at the data point, respectively. The maximum strain achieved at the breakpoint was recorded as EAB, and the percentage was calculated by multiplying with 100.

YM (Pa) was obtained as the initial slope of the tensile stress vs tensile strain graph.

Measurements were performed in three parallels and two repetitions for each sample.

2.3.6 Degree of cross-linking of the films

The cross-linking degree of the films was measured with the TNBS (2,4,6-trinitrobenzene sulfonic acid) assay (Prasertsung et al., 2010). 5 mg of film sample, 1 mL of 0.5% (w/v) TNBS solution, and 1 mL of 4% (w/v) sodium hydrogen carbonate solution were weighed into a glass tube and vortexed. Solutions were heated to 40°C and kept for 2 hours. Then, 2 mL of 6 N HCl was added to the solutions and kept at 60°C for an additional 90 min. In order to prepare the blank sample, the same procedure was applied by adding the HCl solution before the TNBS and $NaHCO_3$ solutions. Lastly, absorbance values were measured at 415 nm in a spectrophotometer (Optizen Pop Uv-Vis, Mecasys, Seoul, Korea). Different

concentrations of glycine were used to prepare the calibration curve, as given in Appendix A (Figure A.1), and the total amounts of free amine groups in the films (μM Glycine/g film) were interpolated from the calibration curve. The degree of crosslinking was calculated by using Equation (11):

$$\text{Degree of crosslinking (\%)} = \frac{\text{Gly}_U - \text{Gly}_C}{\text{Gly}_U} x100 \qquad \text{Equation (11)}$$

where Gly_U and Gly_C are the free amine groups (in μM Glycine/g film) of uncrosslinked and crosslinked samples, respectively. Measurements were performed in three parallels and two repetitions for each sample.

2.3.7 Total phenolic content of the films

Film extracts were prepared before the experiment. 0.5 g of film samples were added to 5 mL 80% (v/v) methanol solution, vortexed for 3 min, and rested for 3 hours. Later, samples were vortexed again for 3 min, filtered, and centrifuged (MIKRO 220R Hettich Zentrifugen, Tuttlingen, Germany) to remove insoluble particles. These film extracts were used for total phenolic content and antioxidant capacity assays.

The total phenolic content (TPC) of the film samples was analyzed using the Folin-Ciocalteu method (Singleton et al., 1999). For the analysis, 0.5 mL of film solution was mixed with 2.5 mL of Folin-Ciocalteu reagent (0.2 N) and vortexed. After keeping still for 3 min, 4 mL of sodium carbonate solution (7.5%, w/v) was added to reduce the reagent. Solutions were stored at room temperature in the dark for 2 hours and lastly, absorbance values were measured at 760 nm using a spectrophotometer. Different concentrations of gallic acid solutions were used to develop the calibration curve, as given in Appendix A (Figure A.2), and the total phenolic content of the films was interpolated as gallic acid equivalence (mg GAE/g film) from the calibration curve. Measurements were performed in three parallels and two repetitions for each sample.

2.3.8 Antioxidant capacity of the films

In order to comprehensively assess the antioxidant capacity of the films, ABTS˙+ and DPPH• assays were used together. The total antioxidant capacities were measured with ABTS˙+ assay, while free radical scavenging abilities were measured with DPPH• assay.

2.3.8.1 ABTS˙+ assay

The total antioxidant activity of the film samples was measured by using ABTS˙+ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay, as described by Nenadis et al. (2004). ABTS˙+ solution was prepared by dissolving the reagent in water to a concentration of 7 mM and then oxidizing it by mixing with an aliquot volume of 2.6 mM potassium persulfate solution. The solution was stored at room temperature in the dark overnight. Before starting the analyses, the absorbance of the solution at 734 nm was measured with a spectrophotometer and adjusted to 1000 by mixing the solution with pure methanol. For the analysis, 300 µL of film extract and 2700 µL of ABTS+ solution were mixed, vortexed, and rested in the dark for 30 minutes. Then, absorbance values were measured at 734 nm in a spectrophotometer. Different concentrations of Trolox solutions were used to develop the calibration curve, as given in Appendix A (Figure A.3), and the total antioxidant capacity of the films was interpolated as Trolox equivalence (mg TE/g film) from the calibration curve. Measurements were performed in three parallels and two repetitions for each sample.

2.3.8.2 DPPH• assay

The free radical scavenging abilities of the film samples were analyzed using the DPPH• (2,2-diphenyl-1-picrylhydrazyl) assay, as Maroufi et al. (2020) described. DPPH• solution was prepared by dissolving the reagent in pure methanol to a

concentration of 6 mM. The absorbance of the solution at 517 nm was adjusted to 1000 by mixing with pure methanol before the analyses. Film samples were prepared by mixing 100 µL of film extract with 3900 µL of DPPH• solution and vortexing. After resting the film samples in the dark for 30 minutes, the absorbance values were measured at 517 nm using a spectrophotometer. DPPH• inhibition (%) values were calculated by using Equation (12):

$$\%\ \text{DPPH} \bullet \text{inhibition} = \frac{A_0 - A_S}{A_S} x100 \qquad \text{Equation (12)}$$

where A_0 is the absorbance of the DPPH• solution and A_s is the absorbance of the film sample. Measurements were performed in three parallels and two repetitions for each sample.

2.3.9 Fourier Transform Infrared (FTIR) spectroscopy of the films

Film samples were analyzed with an FTIR spectrophotometer (IR-Affinity1, Shimadzu Co., Kyoto, Japan) to investigate the chemical interactions between faba bean protein, glycerol, transglutaminase, and thyme oil. Measurements were carried out with 64 scans within the wavelength range of 600-4000 cm^{-1} and in a resolution of 4 cm^{-1}. Samples were analyzed at room temperature, and measurements were performed in three parallels with two repetitions for each sample.

2.3.10 Thermo-gravimetric analysis (TGA) of the films

In order to identify the thermal characteristics of the films, thermo-gravimetric analysis was performed in a TGA instrument (STA 6000, Perkin Elmer, MA, USA). 5 mg of film samples were weighed into a platinum pan and placed under an inert atmosphere with a flow rate of 30 mL N_2/min. Then, samples were heated from 30°C to 600°C at a constant heating rate of 10°C/min. The weight loss of the film samples was recorded as a function of temperature. Thyme oil and faba bean protein concentrate samples were also analyzed as a reference.

2.3.11 X-ray diffraction (XRD) analysis of the films

The crystalline properties of the films were investigated by an X-ray diffractometer (Ultima IV, Rigaku, Tokyo, Japan) with Cu irradiation. The analysis was carried out with 40 kV voltage and 30 mA current. Film samples were scanned in the angular range of 3° and 90° with a scan rate of 2º min^{-1}. Faba bean protein concentrate was also analyzed as a reference.

2.3.12 Antimicrobial activity of the films

The antimicrobial activity of the thyme oil films was tested against Gram (+) *S.aureus* (ATCC 43300) and Gram (−) *E.coli* (ATCC 25922) strains. Frozen colonies were taken from the -80ºC storage chamber and transferred to tryptic soy broth with a sterile inoculation loop. After incubating overnight at 37ºC, the absorbance of each medium was measured at 600 nm wavelength with a spectrophotometer (Optizen Pop Uv-Vis, Mecasys, Seoul, Korea) to ensure the cell density of approximately 10^8 CFU/mL. Then, 100 µL of *E.coli* or *S.aureus* suspensions were transferred to sterile Petri dishes, which were prepared with Tryptic Soy Agar shortly before inoculation and spread with a Drigalski spatula. Films were cut into round shapes with Ø1 cm and placed on inoculated petri dishes. Dishes were incubated at 37ºC for 24 h in order to achieve microbial growth in selective media. Lastly, the diameter of inhibition zones was measured with a digital caliper. All measurements were performed in three parallels and two repetitions for each sample.

2.4 Statistical analysis

Statistical analyses were performed using Minitab 21 (State College, PA, USA). Analysis of variance (ANOVA) was used to evaluate the overall effect of the independent variables (pH, enzyme concentration, and the presence of thyme oil) on the different samples. Tukey's Multiple Comparison Test was subsequently applied

to identify significant differences between samples within a 95% confidence interval. The results were represented as mean values and standard deviations.

CHAPTER 3

RESULTS AND DISCUSSION

This thesis study consists of two stages. In the first stage of the study, film-forming solutions were prepared at pH 6 and 8 and with different dosages (0, 5U, and 10U) of microbial transglutaminase. The ideal amounts of protein concentrate (8%, w/v) and glycerol (2.5%, v/v) in the film-forming solutions, and the enzyme dosages to be tested were decided by performing preliminary experiments. Based on the information provided by the protein supplier, the total protein amount of the film-forming solutions was calculated as 4.32 g per 100 mL film-forming solution. In addition, enzyme dosages were computed using the enzyme activity provided by the enzyme supplier. As an ideal enzyme dosages, 5U and 10U were selected, as it was observed during preliminary experiments that higher enzyme dosages resulted in rapid and irregular cross-linking, preventing the formation of uniform films. In addition, similar results were obtained when enzyme-containing samples were incubated at various temperatures and durations before drying. Therefore, samples were immediately located in the oven and dried after preparation. As a result of comparing the films in terms of physical properties, microstructure, and antioxidant capacity, pH 8 was selected as the ideal pH for the second part of the study.

In the second stage of the study, 1% thyme oil was added to each film-forming solution at pH 8, and the same procedure was followed. Then, the films were evaluated in terms of physical properties, microstructure, and antioxidant capacity, and the results were compared with the films prepared without thyme oil addition. Moreover, the antimicrobial activity of the films was measured against *E. coli* and *S. aureus*.

3.1 Effects of pH and MTGase on the Physicochemical Properties of Faba Bean Protein-Based Films

3.1.1 Thickness, moisture content, water solubility and water vapor permeability

Film samples prepared at pH 6 and 8, and with the addition of varying dosages (0, 5U and 10U) of MTGase were evaluated in terms of thickness, moisture content, water solubility, and water vapor permeability. Results were summarized in Table 3.1.

Table 3.1. Thickness, moisture content, water solubility and water vapor permeability of the films

Film sample	Thickness (mm)	Moisture content (%)	Water solubility (%)	WVP $\times 10^{-11}$ (g/h.mm.Pa)
FB_6	$0.184\pm0.015^{a*}$	12.67 ± 1.09^{b}	34.53 ± 2.18^{a}	2.60 ± 0.17^{a}
FB_6_E5	$0.174\pm0.012^{a,b}$	16.14 ± 1.41^{a}	32.72 ± 2.68^{a}	$2.50\pm0.06^{a,b}$
FB_6_E10	$0.166\pm0.005^{a,b,c}$	16.26 ± 0.66^{a}	32.07 ± 0.44^{a}	$2.33\pm0.16^{a,b,c}$
FB_8	0.155 ± 0.009^{c}	12.58 ± 0.45^{b}	35.26 ± 1.89^{a}	2.08 ± 0.03^{c}
FB_8_E5	0.152 ± 0.005^{c}	$14.28\pm1.18^{a,b}$	35.04 ± 1.49^{a}	$2.30\pm0.06^{a,b,c}$
FB_8_E10	$0.157\pm0.005^{b,c}$	15.41 ± 1.24^{a}	33.53 ± 1.54^{a}	$2.11\pm0.15^{b,c}$

*Different letters in the same column show significant differences ($p < 0.05$).

Film solutions prepared at pH 6 resulted in thicker films than those prepared at pH 8. In addition, it was observed during film preparation that the films formed at pH 6 tend to contract in r-direction as they are dried. In other words, films became smaller in diameter and thicker. It can be explained by the reduced protein unfolding at pH values closer to the isoelectric point of the protein due to less hydrophobic interactions (Kocakulak et al., 2019). These results verify the findings of a previous

study (Saremnezhad et al., 2011), in which increasing the pH of the film-forming solution from 7 to 9 and eventually to 11 resulted in a decrease in thickness in faba bean protein-based films. It was also found that MTGase dosage did not cause a significant change in film thickness. This result verified the findings of a previous study conducted with gelatin-zein films cross-linked with TGase, in which different TGase dosages did not change the thickness of the films (Ahammed et al., 2023). Similar results were also observed in another study, where TGase incorporation did not alter the thickness of the tilapia scale gelatin films incorporated with soy protein isolate (Weng & Zheng, 2015). The thickness of a film is a critical parameter, as it directly affects the barrier and mechanical properties of a film.

The moisture content of the films increased with MTGase addition at both pH 6 and 8. In a recent paper, cross-linking with TGase also increased the moisture content of the soy protein isolate-based films at different pH levels (Kang et al., 2024). In another study, incorporation of TGase increased the moisture content of the gelatin-natamycin fungistatic composite films (Wu et al., 2017), and it was explained by higher levels of hydrogen bonding between water molecules and the protein chains due to the presence of TGase, resulting in more water incorporation into film matrix. In addition, statistical analysis showed that the effect of pH on moisture content is insignificant. Similar results were achieved in a previous study, where increasing the pH of a pea protein isolate-based film-forming solution did not significantly affect the moisture content of the films (Kowalczyk & Baraniak, 2011). Likewise, it was observed in another study that pH change did not cause a significant change in the moisture content of the sesame protein-common vetch starch-based composite films (Ertan et al., 2024).

No significant differences were observed between the water solubility of the film samples prepared at different pH values and different enzyme dosages. Similarly, Díaz et al. (2019) found no significant difference in the water solubility of chickpea flour-based films prepared at pH 7 and pH 10 with a 3% glycerol concentration. Shiku et al. (2003) reported comparable water solubility in fish myofibrillar protein-based films prepared at pH 7 and pH 8, attributing this similarity to the stability of

the protein network within this pH range. In another study, Masamba et al. (2016) observed that zein-oleic acid composite films prepared at pH 6 and pH 8 exhibited similar water solubility when dried at 35ºC, with TGase addition having no significant effect. Likewise, Zheng et al. (2022) found no significant differences in the water solubility of gelatin-beeswax composite films crosslinked with 1%, 2%, and 3% TGase, compared to an uncrosslinked reference. The water solubility of the films plays a crucial role in packaging development since higher solubility may compromise the film's structural integrity in moist environments, which may reduce barrier properties while increasing biodegradability.

A statistically significant difference was found between the WVPs of FB_6 and FB_8, where increasing the pH resulted in a significant decrease in WVP. However, no significant differences were observed among the MTGase-treated films. Similarly, in a previous study conducted on pea protein isolate films (Kowalczyk et al., 2011), increasing the pH resulted in a slight decrease in the WVP of the films. In another study (Gennadios et al., 1993), pea protein isolate and wheat gluten-based films had lower WVPs as the pH moved farther from the protein's isoelectric point. Another study (McHugh et al., 1994) suggested that the difference in WVP at different pHs was caused by partial inhibition of the exchange between thiol groups (-SH) and disulfide bonds (-S-S-) and reduced thiol oxidation reactions in more acidic pHs. These reactions help stabilize the film matrix by forming a denser protein network, creating a less permeable film with more mechanical strength. On the other hand, different MTGase dosages did not result in significant differences in WVP within the pH 6 or pH 8 groups, indicating that enzyme treatment had no significant impact on samples at either pH level. In a study performed on sago starch-fish gelatin films (Al-Hassan & Norziah, 2017), increasing TGase dosages did not significantly affect the WVP of the films. Similar results were achieved with red bean protein-based films (Tang et al., 2011), where increasing MTGase concentrations resulted in similar WVP values.

WVP of the films varied between 2.08 and 2.60 $\times$ 10^{-11} g/h.mm.Pa, and the lowest value was achieved at FB_8. These WVP values are higher than those reported by

Saremnezhad et al. (2011) for faba bean protein-based films, which ranged from 0.612 to 1.044 × 10^{-11} g/h·mm·Pa, depending on the glycerol content and pH of the film-forming solution. Yildiz et al. (2022) also found lower WVP values for faba bean flour-based films, ranging from 1.044 to 1.692 × 10^{-11} g/h·mm·Pa, depending on the degree of cross-linking. Conversely, higher WVPs have been documented for faba bean-based films; for example, Emir et al. (2023) reported values between 251.64 and 470.08 × 10^{-11} g/h·mm·Pa, while Montalvo-Paquini et al. (2014) reported a range of 960 to 1200 × 10^{-11} g/h·mm·Pa. Saremnezhad et al. (2011) explained these variances in WVP values as resulting from different proportions of hydrophobic compounds in the film matrix. Low WVP in food packaging films is crucial since controlling the moisture transfer means preventing the food from drying out or becoming too moist and susceptible to microbial spoilage.

3.1.2 Colour and opacity

Color and opacity are essential parameters for food packaging materials, as they amplify the light exposure of the food, which may accelerate deterioration. The appearance of the package influences consumer appeal. Tables 3.2 and 3.3 summarize the color measurement results of the films prepared at pH 6 and pH 8 with varying dosages of MTGase.

Table 3.2. Colour properties of the films

Film sample	L*	a*	b*	ΔE*
FB_6	95.16±0.66^{a**}	-3.01±0.24^{c}	14.52±2.11^{c}	13.48±2.10^{c}
FB_6_E5	95.16±0.63^{a}	-3.04±0.30^{c}	16.88±1.82^{a,b,c}	15.76±1.87^{b,c}
FB_6_E10	95.76±0.41^{a}	-3.07±0.29^{c}	16.33±1.78^{b,c}	15.15±1.79^{b,c}
FB_8	85.75±1.21^{c}	-1.78±0.38^{a}	19.19±3.11^{a}	21.41±2.10^{a}
FB_8_E5	85.74±5.04^{c}	-2.12±0.50^{a,b}	17.94±1.54^{a,b}	20.62±3.64^{a}
FB_8_E10	89.42±2.22^{b}	-2.43±0.36^{b}	16.67±1.48^{a,b,c}	17.43±1.41^{b}

**Different letters in the same column show significant differences ($p < 0.05$).

Table 3.3. Opacity, whiteness and yellowness indexes of the films

Film sample	Opacity	Whiteness Index (WI)	Yellowness Index (YI)
FB_6	0.21±0.03^{c*}	84.38±2.08^{a}	21.80±3.25^{d}
FB_6_E5	0.20±0.04^{c}	82.17±1.88^{a,b}	25.35±2.86^{c,d}
FB_6_E10	0.18±0.03^{c}	82.84±1.77^{a,b}	24.37±2.70^{c,d}
FB_8	0.91±0.18^{a}	75.88±1.98^{c}	31.92±4.77^{a}
FB_8_E5	0.95±0.38^{a}	76.70±3.83^{c}	30.09±4.03^{a,b}
FB_8_E10	0.65±0.17^{b}	79.98±1.45^{b}	26.63±2.34^{b,c}

*Different letters in the same column show significant differences ($p < 0.05$).

Films prepared at pH 6 had a lighter color with higher L* (lightness) and lower a* (redness), compared to the films prepared at pH 8. In addition, FB_6 exhibited lower b* (yellowness) value compared to FB_8, though the difference between enzyme-added films at different pH levels was not statistically significant. Using the relevant formula, it was calculated that films prepared at pH 6 have lower ΔE* (total color difference), lower opacity, lower YI, and higher WI. However, at 10U enzyme concentration, the differences in ΔE*, YI, and WI between the different pH levels were not statistically significant. These results verified the findings of a previous study conducted with mung bean proteins, in which the pH increase resulted in decreases in the L* and a* values of the films while the b* value increased (Bourtoom, 2008). Saremnezhad et al. (2011) found that increasing the pH from 7 to 9 and 12 in the faba bean protein isolate-based film-forming solution created more significant color differences, resulting in darker and yellower films. In another study on faba bean-based films (Montalvo-Paquini et al., 2014), the main reason for this color change difference was explained as more pigment extraction at higher alkalinity levels.

The addition of MTGase in varying dosages did not cause statistically significant color changes at pH 6. Similarly, at pH 8, adding 5U of MTGase did not change the color parameters notably. On the other hand, adding 10U of MTGase decreased

ΔE*and opacity, while increasing L* and WI. It was reported in another study that the optical response of TGase is not the same at low and high levels of enzyme concentration (Yilmaz et al., 2020). In a previous study, where 3% TGase addition increased L*, reduced b*, ΔE*, and opacity of the gelatin-beeswax composite films but did not affect a* value while not creating a significant difference at lower enzyme levels (Zheng et al., 2022). In a study conducted on chicken meat protein films (Yayli et al., 2017), adding TGase increased the opacity of the films at lower dosages but decreased at higher enzyme levels. Another study verified these findings, where cross-linking with TGase decreased the opacity of the gelatin-zein composite films (Ahammed et al., 2021). These variations in the color and opacity responses were explained by the changes in the molecular structure and crystallinity in the film matrix due to cross-linking, which causes different responses to light (Wangtueai et al., 2010).

3.1.3 Mechanical properties

Food packaging materials need to have good mechanical properties to resist potential damage during handling, transportation, and storage without any deformation. They are essential to protect the food material from external forces, preserve product integrity, and extend shelf life. Tensile strength, % elongation at break, and Young's modulus measurement results of the films prepared at pH 6 and pH 8 with varying dosages of MTGase were given in Table 3.4.

Table 3.4. Mechanical properties of the films

Film sample	Tensile Strength (MPa) (TS)	Elongation at Break (%) (EAB)	Young's Modulus (MPa) (YM)
FB_6	$5.02\pm0.48^{b,c*}$	$8.83\pm2.16^{c,d}$	$189.58\pm18.75^{a,b,c}$
FB_6_E5	3.49 ± 0.82^{d}	5.20 ± 0.78^{d}	137.45 ± 47.01^{c}
FB_6_E10	$3.72\pm0.74^{c,d}$	3.97 ± 0.72^{d}	$169.09\pm27.33^{b,c}$
FB_8	7.03 ± 0.70^{a}	$22.44\pm7.14^{a,b}$	249.15 ± 44.05^{a}
FB_8_E5	$5.73\pm1.02^{a,b}$	$16.32\pm2.76^{b,c}$	$227.57\pm44.47^{a,b}$
FB_8_E10	$5.83\pm0.72^{a,b}$	23.91 ± 5.36^{a}	246.54 ± 28.29^{a}

*Different letters in the same column show significant differences ($p < 0.05$).

In general, films prepared at pH 8 had higher TS, EAB, and YM values than those prepared at pH 6. These results showed that films made at pH 8 can withstand greater force, stretch further under mechanical force, and resist deformation, making these films stronger and more durable. Similar results were reported in a previous study, where increasing the pH from 7 to 9 and eventually 11 increased TS, EAB, and YM of the pea protein isolate films (Kowalczyk et al., 2014). Likewise, increasing the pH farther from the pI increased the TS, EAB%, and YM of the mung bean isolate-based films (Nasriyanti et al., 2009). In another study, TS and EAB% of the peanut protein-based films increased when the pH of the film-forming solutions was increased from 6 to 7.5 and 9 (Jangchud & Chinann, 1999), and these results were explained by increased protein-protein interactions at pHs close to pI, resulting in a denser and less elastic film. Therefore, it can be concluded that films prepared at pH 8 had better mechanical properties.

The effect of MTGase on the YM of the films was statistically insignificant. At pH 8, a statistically significant difference was observed between the EAB values of FB_8_E5 and FB_8_E10 films, though both values remained comparable to FB_8. Yayli et al. (2017) reported a similar trend in deboned chicken meat films, where

films with 1% MTGase had statistically lower EAB than those with 2% MTGase, yet both were comparable to uncross-linked films. Similarly, Kouravand et al. (2020) observed that up to 10U of MTGase decreased the EAB% of whey protein isolate-based films, but EAB began to increase at 15U, attributed to excessive cross-link formation resulting in an insufficient network structure.

The TS results of this study differed from previous findings. In the literature, several studies have found that MTGase incorporation increases TS of films due to enhanced covalent iso-peptide bonding, which strengthens the polymer network (Kouravand et al., 2020). In contrast, in this study, MTGase incorporation had no significant effect on TS at pH 8; at pH 6, however, TS decreased with 5U MTGase but recovered at the 10U dosage. Similar findings were reported by Yilmaz et al. (2020), who studied anchovy by-product-based films at pH 6.7 and found that TS decreased with 2% MTGase but did not differ significantly from the reference at 1% and 3% dosages. The TS decrease in films may be due to excessive enzyme dosage leading to random cross-links and local stiff points, which can create cracks or fractures. Additionally, decreased film mobility due to cross-linking increases brittleness and may reduce the film's ability to withstand stress (Weng & Zheng, 2015)

3.1.4 Degree of cross-linking

The degree of cross-linking analysis was essential to measure the effectiveness of MTGase treatment and explain the changes in the functional properties of the films in different MTGase dosages. The results of the measurements are reported in Table 3.5 as free amine groups (μM Glycine/g film) at each condition. These values indicate the number of unreacted amino groups per gram of film, with higher values implying a lower degree of cross-linking. Additionally, the degree of cross-linking was calculated for each pH individually, using the films made without enzyme addition as the uncrosslinked reference.

Table 3.5. Free amine group content (μM Glycine/g film) and degree of cross-linking of the films

Film sample	Free Amine Group Content (μM Glycine)/g film	Degree of Cross-linking (%)
FB_6	86.41±8.54[a*]	-
FB_6_E5	76.07±17.45[a,b]	9.84±18.16
FB_6_E10	74.05±19.16[a,b]	11.77±19.60
FB_8	83.08±4.87[a,b]	-
FB_8_E5	70.24±11.63[a,b]	12.61±12.18
FB_8_E10	59.27±14.12[b]	23.40±14.36

*Different letters in the same column show significant differences ($p < 0.05$).

After statistical analysis, it was observed that pH variation and different MTGase levels did not significantly affect the free amine group contents of the films when considered independently. The only statistically significant difference was found between FB_6 and FB_8_E10, indicating that the combination of pH adjustment and a high MTGase dosage was effective in producing a well-cross-linked film. These findings align with a previous study on soy protein-based films, where SPI films treated with TGase under different pH conditions (Kang et al., 2024) showed similar trends. Like faba bean protein, the isoelectric point of soy protein isolate is 4.5, and the highest degree of cross-linking was observed in films treated with 10U TGase at pH 8. This effect was attributed to enhanced protein denaturation under alkaline conditions, which exposed additional amino acid residues, increasing the potential for cross-linking. Additionally, other studies in the literature support the observation that higher TGase dosages enhance the degree of cross-linking. For instance, in gelatin-based films, increasing MTGase incorporation at pH 8.5 led to higher degrees of cross-linking (Sabaghi et al., 2022).

3.1.5 Antioxidant properties and total phenolic content

The antioxidant capacity and total phenolic content of the films prepared at pH 6 and 8 are summarized in Table 3.6.

Table 3.6. Antioxidant properties and total phenolic content of the films

Film sample	Total Phenolic Content (mg GAE/g film)	ABTS˙+ Radical Scavenging Activity (μmol TEAC/g film)	DPPH· Inhibition (%)
FB_6	2.73±0.36^{b,c*}	755.08±45.19^{a}	103.40±9.74^{b}
FB_6_E5	3.33±0.38^{a,b}	700.79±43.86^{a,b}	128.59±13.94^{a}
FB_6_E10	3.65±0.34^{a}	763.50±94.95^{a}	132.18±18.14^{a}
FB_8	2.21±0.26^{c}	586.76±19.90^{b}	36.20±3.01^{c}
FB_8_E5	2.59±0.22^{c}	642.57±71.56^{a,b}	41.84±4.76^{c}
FB_8_E10	2.56±0.33^{c}	584.34±82.20^{b}	41.78±5.68^{c}

*Different letters in the same column show significant differences ($p < 0.05$).

Films prepared at pH 6 generally exhibited higher antioxidant activity and had higher TPC than those made at pH 8. These results confirmed the findings of a study conducted on modified quinoa protein films, in which pH increase caused a reduction in TPC and % DPPH· inhibition (Mir et al., 2023). It was explained with the detrimental effect of alkalinity on antioxidant activity due to the degradation of phenolic compounds. Likewise, it was reported in another study that increasing alkalinity caused a significant reduction in ABTS˙+ radical scavenging activity, % DPPH· inhibition, and TPC of the gallic acid enriched sesame protein-common vetch starch-based films (Ertan et al., 2024). The observed reductions were attributed to the deprotonation of phenolic groups, which diminishes their ability to donate protons and decreases their effectiveness in neutralizing free radicals.

Cross-linking with MTGase slightly increased the TPC and % DPPH· inhibition of the films at pH 6. However, the effects of cross-linking on ABTS˙+ radical

scavenging activity were statistically insignificant. Moreover, cross-linking did not significantly change the TPC and antioxidant properties of the films prepared at pH 8. Similar results were observed in a previous study, where MTGase incorporation increased % DPPH· inhibition of the lactoferrin-*Tremella fuciformis* solutions (Wang et al., 2024b). Likewise, TGase treatment increased the antioxidant performance of ovalbumin solutions by enhancing the % DPPH· inhibition (Zhu et al., 2024). This increase was attributed to the reorganization of the protein matrix due to TGase treatment, which may result in the exposure of hydrophobic regions within the protein chain, making the protein more capable of interacting with free radicals, thereby enhancing antioxidant activity.

3.1.6 Fourier Transform Infrared (FTIR) spectroscopy

In order to explain the intermolecular interactions and structural changes that took place in the film, FTIR analysis was performed. FTIR spectra of the raw materials are given in Appendix B (Figure B.1.) and FTIR spectra of the films at pH 6 and pH 8 with varying dosages of MTGase are shown in Figure 3.1.

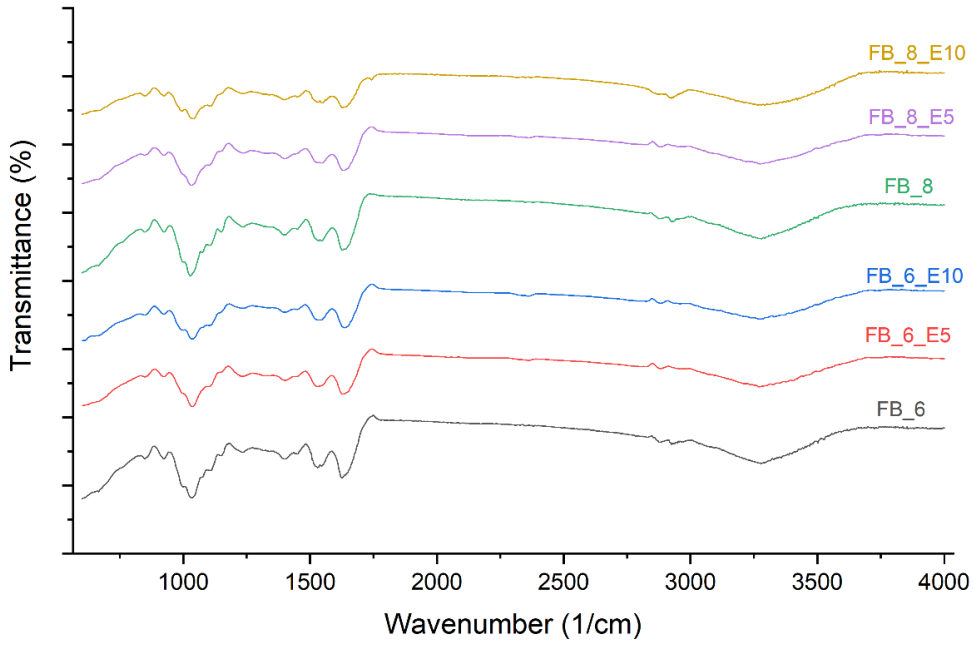


Figure 3.1. FTIR Spectra of the films at pH 6 and pH 8

When studying MTGase cross-linked faba bean-based protein films, the key wavenumbers to analyze are the amide I band (1600–1650 cm^{-1}), which primarily reflects C=O and C-N stretching vibrations and shows changes in hydrogen bonding and protein folding, and the amide II band (1480–1575 cm^{-1}), which corresponds to C-N stretching and N-H bending, revealing structural details related to protein backbone interactions and folding patterns. Additionally, the amide III band around 1200–1250 cm^{-1}, indicating N-H and C-N vibrations, and a broad band around 3250–3650 cm^{-1}, indicating hydrogen bonding, may also provide helpful information (Bunaciu et al., 2014; Nandiyanto et al., 2019).

No structural differences were observed between samples when comparing the films prepared at pH 6 and pH 8 without enzyme addition. Similarly, films prepared at pH 6 and pH 8 with 5U MTGase showed the same spectral profile. However, in the 10U enzyme-added films, FTIR analysis showed significant structural changes between pH 6 and pH 8. At pH 8, slightly broader peaks were achieved around 3275 cm^{-1}

and between 1300–1400 cm^{-1} due to enhanced O-H stretching vibration, which was attributed to the formation of inter-molecular and intra-molecular hydrogen bonds (Wang et al., 2019), and higher amounts of bound water (Al-Hassan & Norziah, 2017). The new peaks around 1743 cm^{-1}, indicating C=O stretching, and around 2927 cm^{-1}, signifying asymmetric -CH stretching of $-CH_2$ groups, confirmed the enhanced cross-linking and increased hydrophobic interactions, respectively (Gutiérrez, 2017). At pH 6, a distinct peak was observed at 1540 cm^{-1}, which was attributed to N–H bending and C–N stretching vibrations, and this peak was explained with a lower degree of cross-linking around the amide II band (Ahammed et al., 2021). In addition, a peak shift was observed in the amide I band from 1627 cm^{-1} at pH 6 to 1635 cm^{-1} at pH 8, signifying increased hydrogen bonding in the C=O groups (Núñez-Flores et al., 2013). Since the amide I band is related to the secondary structures of the proteins, this peak shift was also explained by a change in the amount of random coil structure (Zheng et al., 2022).

Similar to the effect of pH, increasing MTGase dosages resulted in broader bands around 3275 cm^{-1} at both pH 6 and pH 8, implying more bound water in the film network. The peak around 1538 cm^{-1} decreased with higher MTGase dosages, especially at pH 8, suggesting stronger cross-linking at this pH. Similarly, the peak at 1743 cm^{-1} appeared only in films with 10U MTGase at pH 8, further indicating higher levels of cross-linking under these conditions. The intensity at 2927 cm^{-1} decreased with 5U MTGase at both pH levels but increased again with 10U MTGase at pH 8 while remaining steady at pH 6. This pattern suggests that hydrophobicity initially decreases as cross-linking increases, but hydrophobicity is restored with higher cross-linking at pH 8 with 10U MTGase. Likewise, the peak intensity at 1450 cm^{-1}, associated with the CH_3 bending modes of the methyl groups in proteins, decreased with 5U MTGase but increased again with 10U MTGase, with this rise being more apparent at pH 8. This change and the band at 3275 cm^{-1} support the noticed hydrophobicity pattern related to enzyme dosage (Nandiyanto et al., 2019). Additionally, the rise in intensity at 2849 cm^{-1} was observed only with 10U

MTGase at pH 8 and corresponds to asymmetric C-H stretching vibrations, serving as another indicator of hydrophobicity (Zheng et al., 2022).

In the spectra's fingerprint region, the bands' intensities around 1150 cm^{-1} and 1075 cm^{-1} slightly decreased with increasing MTGase dosage. These wavelengths correspond to C-C and C-O stretches and are primarily associated with glycerol (Leceta et al., 2018).

3.1.7 Thermo-gravimetric analysis (TGA)

Thermal stability is essential in food packaging since it enables a material to withstand temperature changes without losing integrity, ensuring continued protection of the food material. In order to measure the thermal stability of the films, thermogravimetric analysis (TGA) was performed. TGA thermograms and derivative weight (DTG) curves of the films at pH 6 and 8 with varying dosages of MTGase are shown in Figures 3.2 and 3.3, respectively.

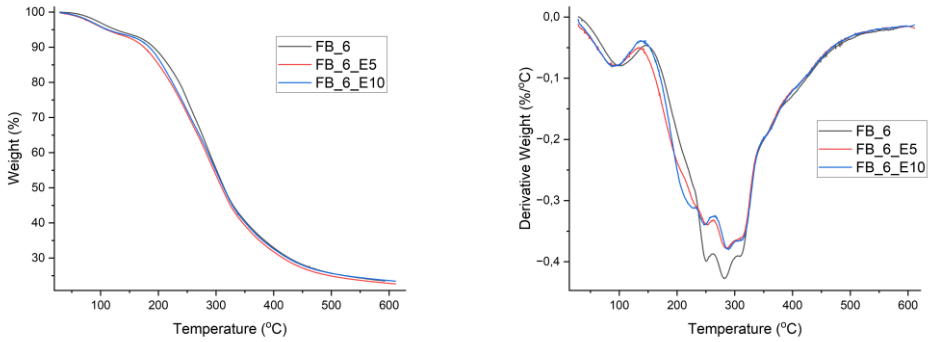


Figure 3.2. TGA thermograms and DTG curves of the films at pH 6

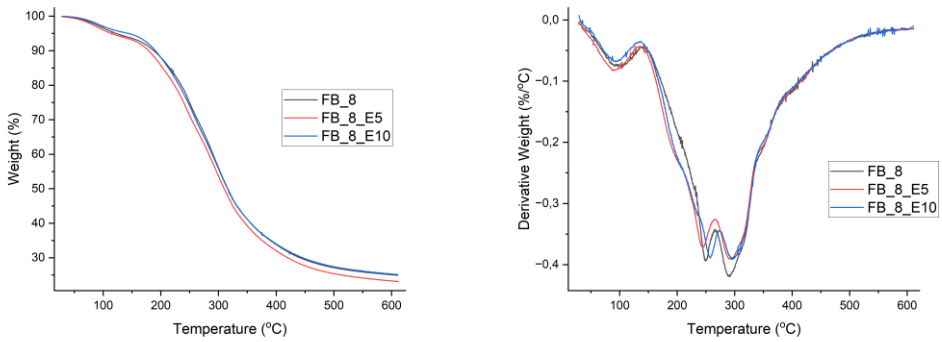


Figure 3.3. TGA thermograms and DTG curves of the films at pH 8

The film's weight loss at pH 6 occurred in four stages. The first stage, from 25 to 125 °C, showed maximum degradation around 100 °C, attributed to water evaporation (Gutiérrez, 2017). The addition of MTGase shifted this peak to 90 °C and slightly increased the total weight loss, likely due to higher moisture content in the enzyme-treated films, which led to a greater proportion of weight loss. The second stage, between 125 and 250 °C, peaked near 250 °C due to glycerol degradation (Rojas-Lema et al., 2023). An additional peak appeared at 227 °C with the incorporation of 10U of MTGase, possibly resulting from phase separation caused by the higher MTGase content disrupting the film matrix (Maroufi et al., 2020). The third stage, from 250 to 350 °C, exhibited two peaks: one at 285 °C, attributed to low molecular weight proteins, and another at 310 °C, associated with higher molecular weight proteins (Mirpoor et al., 2024). The final stage, occurring above 350 °C, involves the breakdown of carbon residues formed during earlier stages, leading to the complete degradation of the protein film (Azevedo et al., 2017). MTGase addition did not produce a notable change in the last stage, and the residual weight of the samples at 600 °C ranged from 22.83% to 23.61%.

At pH 8, the maximum degradation temperatures were similar to those at pH 6, though the addition of MTGase had distinct effects on thermal stability. As with pH 6, the first peak at 100 °C shifted to 90 °C with enzyme addition. Despite the higher moisture content of FB_8_E10, its weight loss at the end of the first stage was lower

than that of the other samples. This result can be attributed to MTGase providing additional bonding sites for water, facilitating the formation of a stable network structure, and preventing water evaporation (Cheng et al., 2019). The second peak in the derivative thermal degradation curve appeared at 250 °C, then decreased to 245 °C with adding 5U of MTGase, and subsequently increased to 257 °C with 10U of enzyme. Since the second peak represents glycerol degradation, the initial decrease can be attributed to reduced interaction between glycerol and the protein matrix, allowing for the formation of higher molecular weight proteins (Liu et al., 2016). Similarly, the increase in the second peak was due to higher levels of cross-linking, which enhanced intramolecular interactions and led to a denser network with greater thermal stability (Mirpoor et al., 2024). Unlike at pH 6, the increased solubility of proteins at pH 8 caused the degradation of all protein sizes to converge into a single peak around 290 °C, likely due to the breakdown of larger protein molecules at pH 8. This breakdown shifted the peak initially observed at 310 °C towards 285 °C, with both peaks ultimately merging at 290 °C. Additionally, this newly formed peak increased to 296 °C with higher MTGase dosages, as the enzyme promoted covalent bond formation, strengthening the protein network and enhancing thermal stability (Liu et al., 2016). Finally, the thermal degradation behavior above 350 °C was similar to that observed at pH 6, with residual weights at 600 °C ranging between 23.28% and 25.28%.

3.1.8 X-ray diffraction (XRD) analysis

Crystallinity measurement is a key indicator for better understanding the internal structure of the films and the arrangement of the molecules constituting the packaging film. For this reason, XRD analysis was performed on the films. XRD pattern of the faba bean protein concentrate is given in Appendix B (Figure B.2.), and the patterns of the films at pH 6 and pH 8 with varying dosages of MTGase are shown in Figure 3.4.

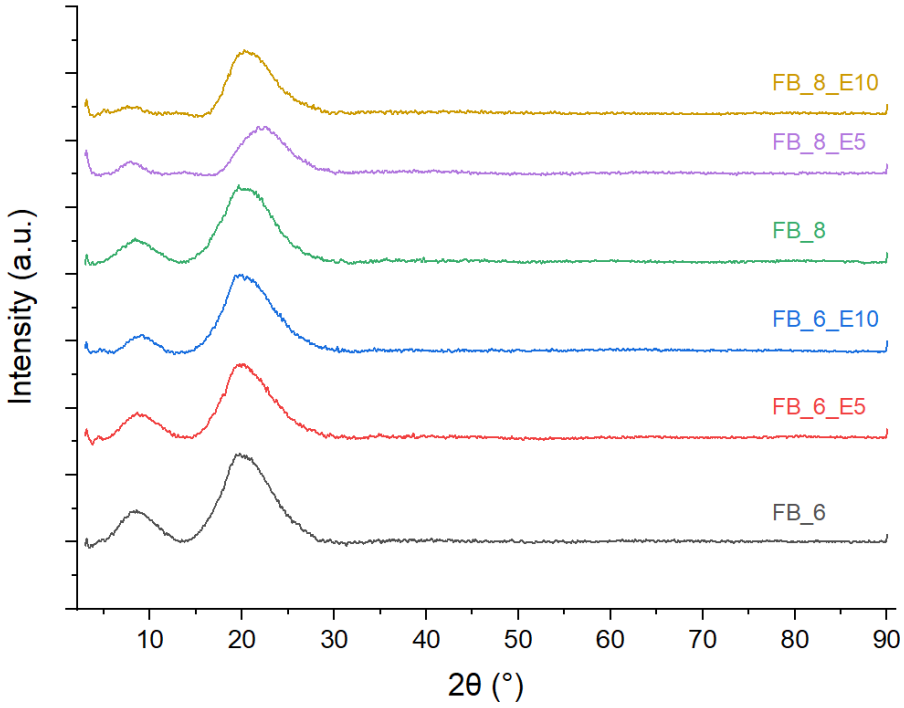


Figure 3.4. XRD patterns of the films at pH 6 and pH 8

Faba bean protein concentrate exhibited two broad peaks in its XRD pattern: one at 2θ = 9.06°, corresponding to the presence of α-helices, and another sharper peak at 2θ = 20.08°, attributed to β-sheets (Wang et al., 2024a). After processing the protein into films, the intensities of both peaks decreased significantly due to protein denaturation during heat treatment, which caused unfolding of the protein structure (Mir et al., 2021). Similar to the protein concentrate, the FB_6 film displayed two main peaks at 2θ = 8.65° and 2θ = 19.72°, with a crystallinity index of 34.5%. The incorporation of MTGase slightly reduced the intensities of both peaks, and the crystallinity index of the films decreased gradually to 32.9% with increasing enzyme dosages, indicating an overall amorphous structure in all films. These findings are consistent with a previous study in which TGase incorporation decreased the peak intensities of gelatin-zein composite films (Ahammed et al., 2021). Similarly, in another study, it was reported that TGase treatment reduced the peak intensities and crystallinity of soy protein isolate-whey protein composite films, and these changes

were attributed to the alterations in the secondary structure of the proteins (Liu et al., 2023). In addition, the first peak in the FB films gradually shifted to 2θ = 9.22° with increasing enzyme dosages. Combined with the decrease in the peak intensities, these shifts can be explained with the formation of new chemical bonds, which disrupted the regular arrangement of the proteins and led to the formation of smaller crystals (Yu et al., 2015). Notably, MTGase cross-linking did not result in the formation of new peaks or the loss of existing ones.

At pH 8, the peak positions were largely preserved, appearing at 2θ = 8.32° and 2θ = 19.62°. However, the intensities of both peaks decreased slightly, and the crystallinity index of the FB_8 film was calculated as 31.2%, suggesting a more amorphous structure at pH 8. This result can be attributed to increased protein denaturation and unfolding at pH 8, which likely disrupted the regular arrangements of α-helices and β-sheets (Mir et al., 2021). Similar to the observations at pH 6, the intensity of the first peak decreased slightly with increasing MTGase dosages. The addition of 5U MTGase caused a significant shift of the second peak to a higher diffraction angle (2θ = 22.54°) and a reduction in intensity, indicating smaller crystal sizes (Sahni et al., 2020). With the incorporation of 10U MTGase, the second peak returned closer to its original position (2θ = 20.26°), and its intensity increased compared to FB_8_E5 but remained lower than FB_8. These findings are consistent with a previous study, where TGase treatment reduced the peak intensities of soy protein isolate films, which was attributed to good compatibility between the enzyme and the protein (Zadeh et al., 2018). The crystallinity index of the films decreased to 29% with 5U MTGase but increased slightly to 30.1% with 10U MTGase.

3.2 Effects of MTGase and Thyme oil on the Physicochemical Properties of Faba Bean Protein-Based Films

3.2.1 Thickness, moisture content, water solubility and water vapor permeability

Film samples prepared at pH 8, with and without thyme oil addition, and with varying dosages (0, 5U and 10U) of MTGase were evaluated in terms of thickness, moisture content, water solubility, and water vapor permeability. Results are summarized in Table 3.7.

Table 3.7. Thickness, moisture content, water solubility and water vapor permeability of the films

Film sample	Thickness (mm)	Moisture content (%)	Water solubility (%)	WVP $\times 10^{-11}$ (g/h.mm.Pa)
FB_8	$0.155\pm0.009^{c*}$	12.58 ± 0.45^{b}	$35.26\pm1.89^{b,c}$	2.08 ± 0.03^{a}
FB_8_E5	0.152 ± 0.005^{c}	$14.28\pm1.18^{a,b}$	$35.04\pm1.49^{b,c}$	2.30 ± 0.06^{a}
FB_8_E10	$0.157\pm0.005^{b,c}$	15.41 ± 1.24^{a}	33.53 ± 1.54^{c}	2.11 ± 0.15^{a}
FB_8_T	0.170 ± 0.004^{a}	$14.35\pm1.59^{a,b}$	38.64 ± 0.52^{a}	2.26 ± 0.09^{a}
FB_8_E5_T	$0.168\pm0.008^{a,b}$	15.76 ± 1.11^{a}	$36.50\pm1.43^{a,b}$	2.25 ± 0.11^{a}
FB_8_E10_T	$0.164\pm0.007^{a,b,c}$	15.99 ± 0.41^{a}	$35.93\pm0.74^{b,c}$	2.33 ± 0.15^{a}

*Different letters in the same column show significant differences ($p < 0.05$).

Thyme oil addition resulted in thicker films than those prepared without thyme oil, and increasing MTGase dosages did not cause a significant difference in film thickness. Similar results were obtained in a previous study, where adding varying levels of essential oils increased the average thickness of chitosan-starch-based films (Perdones et al., 2016). In another study, increasing dosages of thyme oil resulted in an increase in thickness in gelatin-based films (Rahimi et al., 2023). This increase

was explained by the molecular interactions between protein chains and essential oils disrupting the formation of a compact network and enhancing the spatial distance between polymer chains (Tongnuanchan et al., 2013).

In general, thyme oil-added films had higher moisture contents compared to FB_8 film. However, within each MTGase level, the differences in moisture content between films with and without thyme oil were not significant. Additionally, varying MTGase dosages did not affect the moisture content of the thyme oil-added films. These results align with a previous study, which found that increasing thyme oil content up to 1.5% (v/v) did not significantly alter the moisture content of quince seed mucilage films (Jouki et al., 2014). Similarly, another study reported that incorporating thyme oil nano-emulsion did not affect the moisture content of tamarind starch-whey protein concentrate composite films up to 15% (v/v) (Ghoshal & Shivani, 2022).

The addition of thyme oil increased the water solubility of the films without MTGase but did not significantly affect the water solubility of MTGase-incorporated films. Similar results were observed in a study on chitosan-based films, where the addition of thyme oil up to 1% (v/v) increased film solubility. This increase was attributed to essential oil incorporation disrupting the film network, allowing faster water penetration (Hosseini et al., 2009). Another study by Sedlaříková et al. (2019) confirmed this observation, also finding that thyme oil addition increased the solubility of chitosan-based films. Additionally, 10U of MTGase incorporation decreased the water solubility of thyme oil-added films where 5U of MTGase did not create a significant difference. These results confirmed the findings of a previous study, where MTGase incorporation reduced the water solubility of the gelatin-zein composite films enriched with green tea polyphenols (Ahammed et al., 2023). In a prior study by Ahammed et al. (2021), the reduction in water solubility upon cross-linking with TGase was attributed to increased hydrophobicity resulting from the changes in the α-helical structure of the proteins.

Film samples containing thyme oil exhibited similar WVP values to those without thyme oil, and the effects of MTGase dosage on WVP were statistically negligible. These findings are consistent with a previous study on soy protein isolate-based films enriched with thyme oil, where up to 1.2% (w/w) enrichment did not significantly alter the films' WVP (Liu et al., 2024). Similarly, when 0.78% (v/v) thyme oil was added to gelatin-based films, the WVP of films made with steam-exploded gelatin did not significantly change (Rigueto et al., 2024). However, these results contrast with findings from some studies in the literature, such as those on hake protein-based films (Pires et al., 2011) and chitosan-based films (Perdones et al., 2016), where thyme oil incorporation decreased WVP due to increased hydrophobicity.

3.2.2 Colour and opacity

The color measurement results of the films prepared with thyme oil are summarized in Tables 3.8 and 3.9.

Table 3.8. Colour properties of the films

Film sample	L*	a*	b*	ΔE*
FB_8	$85.75\pm1.21^{b**}$	-1.78 ± 0.38^{c}	$19.19\pm3.11^{c,d}$	21.41 ± 2.10^{c}
FB_8_E5	85.74 ± 5.04^{b}	$-2.12\pm0.50^{c,d}$	$17.94\pm1.54^{d,e}$	$20.62\pm3.64^{c,d}$
FB_8_E10	89.42 ± 2.22^{a}	-2.43 ± 0.36^{d}	16.67 ± 1.48^{e}	17.43 ± 1.41^{d}
FB_8_T	$78.88\pm2.46^{c,d}$	-1.35 ± 0.25^{b}	21.81 ± 1.09^{b}	27.57 ± 2.43^{b}
FB_8_E5_T	79.69 ± 2.67^{c}	-1.26 ± 0.15^{b}	$21.20\pm2.29^{b,c}$	26.55 ± 3.42^{b}
FB_8_E10_T	75.45 ± 2.48^{d}	-0.80 ± 0.13^{a}	24.34 ± 1.72^{a}	31.73 ± 2.88^{a}

**Different letters in the same column show significant differences ($p < 0.05$).

Table 3.9. Opacity, whiteness and yellowness indexes of the films

Film sample	Opacity	Whiteness Index	Yellowness Index
FB_8	$0.91\pm0.18^{c,d*}$	75.88 ± 1.98^{b}	31.92 ± 4.77^{c}
FB_8_E5	0.95 ± 0.38^{c}	$76.70\pm3.83^{a,b}$	$30.09\pm4.03^{c,d}$
FB_8_E10	0.65 ± 0.17^{d}	79.98 ± 1.45^{a}	26.63 ± 2.34^{d}
FB_8_T	1.29 ± 0.13^{b}	69.59 ± 2.45^{c}	39.59 ± 3.20^{b}
FB_8_E5_T	1.32 ± 0.20^{b}	70.61 ± 3.43^{c}	38.17 ± 5.30^{b}
FB_8_E10_T	1.61 ± 0.18^{a}	65.41 ± 2.89^{d}	46.23 ± 4.59^{a}

*Different letters in the same column show significant differences ($p < 0.05$).

Thyme oil incorporation decreased L* and WI of the films while increasing their a*, b*, ΔE*, opacity, and YI. A previous study (Perdones et al., 2016) found that thyme oil incorporation decreased the L* and WI while increasing the ΔE* of the chitosan-based films. Another study verified these results since increasing dosages of thyme oil were found to decrease the L* and WI and to increase the b*, ΔE*, and YI of the quince seed mucilage-based films (Jouki et al., 2014). The color change was explained by phenolic compounds in thyme oil, which could absorb light at low wavelengths. In a study conducted with sodium caseinate-based films, clove oil incorporation decreased the WI and increased the opacity of the films (Xu et al., 2024). Likewise, another study reported that increasing dosages of thyme oil decreased the L* and WI but increased the b*, ΔE*, YI, and opacity of the soy protein-based films (Liu et al., 2024). The increase in opacity was explained by the presence of oil droplets distributed in the film network, which scattered the light and reduced the light transmission through the film.

Cross-linking the thyme oil-added films with MTGase did not create a significant difference at 5U dosage, but decreased WI and increased a*, b*, ΔE*, opacity, and YI at 10U concentration. The effects of increasing dosages of MTGase verified the findings of another study, in which gelatin-zein composite films were cross-linked with TGase and enriched with green tea polyphenols (Ahammed et al., 2023). In this study, high TGase levels resulted in similar L* levels while increasing the a*, b*,

ΔE*, and opacity, leading to lower WI and higher YI. Similar results were achieved in another study, where cardoon seed oil cake was used as the main component of the packaging film, and the effects of MTGase were investigated (Mirpoor et al., 2024). In this study, SEM images verified the presence of oil droplets within the film matrix, and increasing dosages of MTGase resulted in a decrease in L* and increases in a*, b*, ΔE*, and opacity, leading to lower WI and higher YI. The changes in color and opacity were explained by the formation of crystalline regions within the film matrix due to iso-peptide bonds and protein aggregation caused by cross-linking. The reduction in amorphous regions altered the light transmission through the film, changing both the opacity and color of the film. In addition, as seen in the results of the cross-linking analysis, it can be concluded that the presence of thyme oil reduced the cross-linking and created an optical response similar to the low level of TGase addition explained in the first part of the study.

3.2.3 Mechanical properties

The mechanical properties of the thyme oil-added films are summarized in Table 3.10.

Table 3.10. Mechanical properties of the films

Film sample	Tensile Strength (MPa) (TS)	Elongation at Break (%) (EAB)	Young's Modulus (MPa) (YM)
FB_8	7.03±0.70^{a*}	22.44±7.14^{a,b}	249.15±44.05^{a}
FB_8_E5	5.73±1.02^{b}	16.32±2.76^{a,b}	227.57±44.47^{a,b}
FB_8_E10	5.83±0.72^{a,b}	23.91±5.36^{a}	246.54±28.29^{a}
FB_8_T	5.82±0.33^{a,b}	22.12±3.74^{a,b}	208.93±14.28^{a,b}
FB_8_E5_T	5.12±0.41^{b,c}	17.91±5.09^{a,b}	202.28±22.67^{a,b}
FB_8_E10_T	4.31±0.49^{c}	13.55±2.88^{b}	169.94±26.52^{b}

*Different letters in the same column show significant differences ($p < 0.05$).

Thyme oil incorporation decreased TS, EAB and YM of the films in 10U MTGase added films, significantly. These results verified the findings of a previous study, where increasing thyme oil concentrations reduced the TS, EAB, and YM of the composite films made with whey protein isolate and cellulose nanofibers (Carvalho et al., 2020). These reductions were attributed to the partial disruption of interactions between polymer chains caused by the presence of non-polar lipid molecules, leading to a more elastic film with reduced structural support and in another study on starch-gellan films, 0.5 g thyme oil addition per g of polymer resulted in a decrease in the TS, EAB, and YM of films prepared with different ratios of starch, gellan, and lecithin (Sapper et al., 2018). However, low dosages of thyme oil were observed to act as a plasticizer and increase the EAB of the films. Therefore, it is vital to highlight the dosage-dependent nature of essential oils' effects on film properties.

Like the films prepared at pH 8 without the addition of thyme oil, increasing MTGase dosages decreased the TS, EAB, and YM of the thyme oil-added films. However, the effect of excessive cross-linking is not observed in thyme oil-added films. As measured in the degree of cross-linking analysis, thyme oil addition reduced the cross-linking of the films, which may have prevented the random cross-link formation.

3.2.4 Degree of cross-linking

The degree of cross-linking measurement results of thyme oil-added films are summarized in Table 3.11.

Table 3.11. Free amine groups content (μM Glycine/g film) and degree of cross-linking of the films

Film sample	Free Amine Groups Content (μM Glycine)/g film	Degree of Cross-linking (%)
FB_8	83.08±4.87^{a,b*}	-
FB_8_E5	70.24±11.63^{b,c}	12.61±12.18
FB_8_E10	59.27±14.12^{c}	23.40±14.36
FB_8_T	89.14±8.62^{a}	-
FB_8_E5_T	83.97±3.53^{a,b}	4.80±8.29
FB_8_E10_T	79.51±1.87^{a,b}	8.94±7.49

*Different letters in the same column show significant differences ($p < 0.05$).

Thyme oil addition resulted in significantly higher level of free amine groups and lower degree of cross-linking when 10U of MTGase was added. The effects of thyme oil at other enzyme dosages (0 and 5U) were found to be statistically insignificant. Similar results were reported in a previous study where green tea polyphenols were incorporated into TGase-treated gelatin-zein composite films (Ahammed et al., 2023). It was observed that increasing MTGase dosages enhanced the degree of cross-linking, while higher levels of green tea polyphenols reduced the degree of cross-linking at the same enzyme levels. The effects of enzyme dosage was explained with higher number of reactions occurring within the same time frame at higher enzyme levels (Al-Hassan & Norziah, 2017). In another study, where calcium carbonate was used as a cross-linking agent in alginate films, the incorporation of oregano essential oil interfered with the cross-linking mechanism. It reduced the intermolecular forces between polymer chains (Benavides et al., 2012). Therefore, the decrease in the cross-linking with thyme oil addition could be related to oil droplets in the film matrix, reducing the interaction between protein chains (Tongnuanchan et al., 2016).

3.2.5 Antioxidant properties and total phenolic content

TPC, $ABTS^{\cdot+}$ radical scavenging activity, and % $DPPH^{\cdot}$ inhibition measurements of the thyme oil added films are summarized in Table 3.12.

Table 3.12. Antioxidant properties and total phenolic content of the films

Film sample	Total Phenolic Content (mg GAE/g film)	$ABTS^{\cdot+}$ Radical Scavenging Activity (μmol TEAC/g film)	$DPPH^{\cdot}$ Inhibition (%)
FB_8	2.21±0.26^{c*}	586.76±19.90^{b}	36.20±3.01^{b}
FB_8_E5	2.59±0.22^{c}	642.57±71.56^{b}	41.84±4.76^{b}
FB_8_E10	2.56±0.33^{c}	584.34±82.20^{b}	41.78±5.68^{b}
FB_8_T	13.71±0.90^{a,b}	980.79±23.91^{a}	67.69±6.45^{a}
FB_8_E5_T	14.80±1.51^{a}	992.67±13.16^{a}	72.10±8.32^{a}
FB_8_E10_T	13.30±0.53^{b}	973.71±24.68^{a}	69.94±3.11^{a}

*Different letters in the same column show significant differences ($p < 0.05$).

The addition of thyme oil significantly increased the TPC and antioxidant properties of the films, attributed to the high phenol content naturally present in thyme oil, with a strong correlation observed between TPC and antioxidant activity (Aljabeili et al., 2018). On the other hand, changes in the MTGase dosage did not significantly affect the TPC and antioxidant capacity of the films. A previous study reported similar findings, where changes in TGase dosage of tea polyphenol-enriched gelatin-zein films caused fluctuations in $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radical scavenging activities, but these changes were not statistically significant in affecting the antioxidant properties (Ahammed et al., 2023).

3.2.6 Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectra of the thyme oil-added films at pH 8 with varying dosages of MTGase are shown in Figure 3.5.

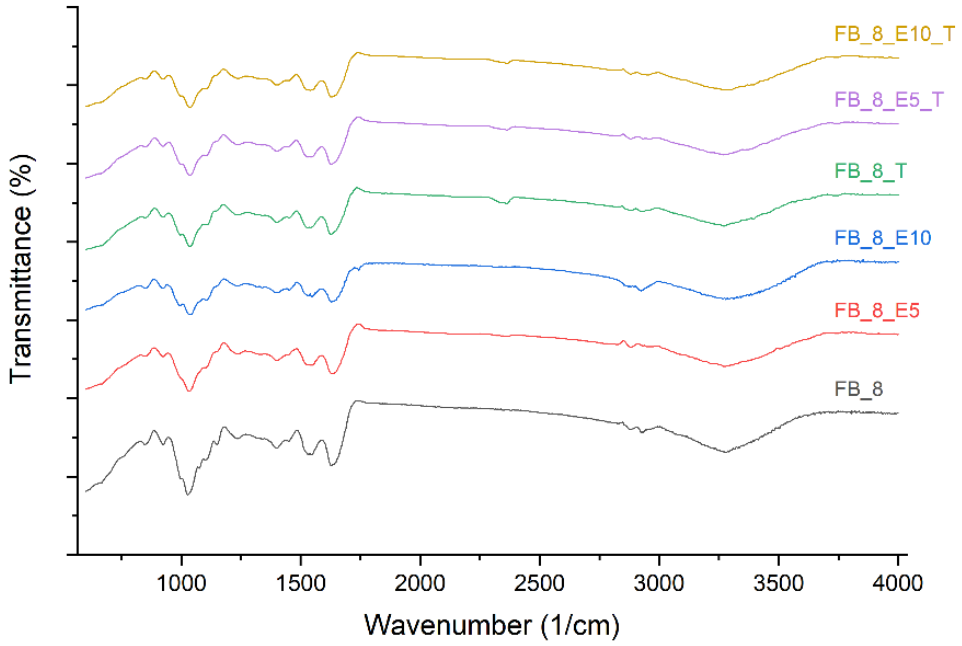


Figure 3.5. FTIR Spectra of the thyme oil added films

Adding thyme oil at pH 8 resulted in a small, broad peak formation at 3394 cm^{-1}, with decreasing intensity at higher MTGase dosages, associated with O–H and N–H bonds in the amino group. This peak suggests the involvement of NH groups and thyme oil in hydrogen bonding (Dang et al., 2015; Sedlaříková et al., 2019). The peak around 3270 cm^{-1} became broader, indicating an increase in bound water in the system (Al-Hassan & Norziah, 2017). The peak near 2945 cm^{-1}, representing the asymmetric stretch of CH_2 , decreased in intensity with thyme oil addition and shifted to 2938 cm^{-1} as MTGase levels increased, further reducing its intensity. These changes suggest interactions between thyme oil and the film matrix, forming inter- and intramolecular bonds (Ocak, 2020). The peaks around 1145 cm^{-1},

corresponding to C–C and C–O stretches associated with glycerol (Leceta et al., 2018), and around 1237 cm^{-1}, indicating C–N stretching and N–H deformation and signaling the presence of hydrogen bonds (Dang et al., 2015), also decreased with the addition of thyme oil; however, MTGase dosage did not produce a notable difference.

3.2.7 Thermo-gravimetric analysis (TGA)

Figure 3.6 shows TGA thermograms and derivative weight (DTG) curves of the thyme oil-added films at pH 8 with varying dosages of MTGase.

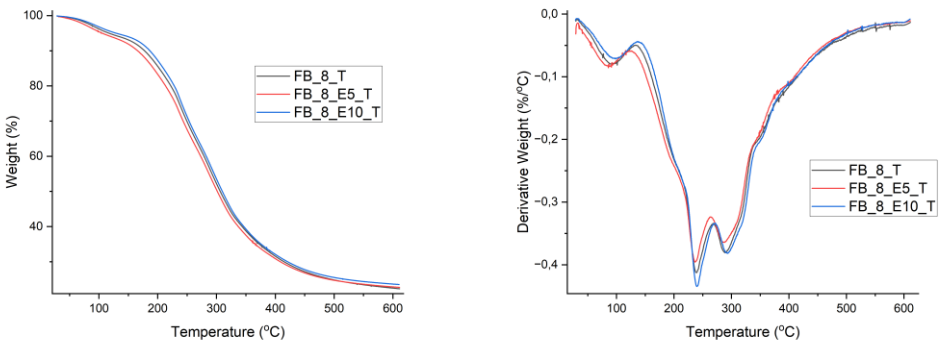


Figure 3.6. TGA thermograms and DTG curves of the thyme oil added films

Thyme oil incorporated films showed a comparable thermal behavior to pH 8 films across all MTGase dosages, with minor negative shifts in peak temperatures. The initial peak occurred at 95°C, decreasing to 85°C with adding 5U of MTGase and subsequently increasing to 100°C with 10U of MTGase. The second thermal peak was observed at 239°C, slightly reduced to 237°C with 5U of MTGase, and elevated to 240°C with 10U of MTGase. The final peak appeared at 288°C, shifting to 285°C with 5U of MTGase and then rising to 292°C with 10U of MTGase. Similar results were achieved in a previous study, where thyme oil incorporation caused a negative shift in the TGA and DTG curves of the gelatin-based films (Rigueto et al., 2024).

Likewise, varying levels of ginger, turmeric, and plai oils gave similar results in fish skin gelatin-based films, where depending on the type and dosage of the oil, the TGA and DTG curves of the films were slightly shifted (Tongnuanchan et al., 2013). These shifts were explained by the interruption of protein-protein interactions due to the presence of oils causing discontinuities in the film matrix. Lastly, residual weights of the films at 600 °C ranged between 22.50% and 23.50%.

3.2.8 X-ray diffraction (XRD) analysis

Figure 3.7 shows the XRD patterns of the thyme oil-added films at pH 8 with varying dosages of MTGase.

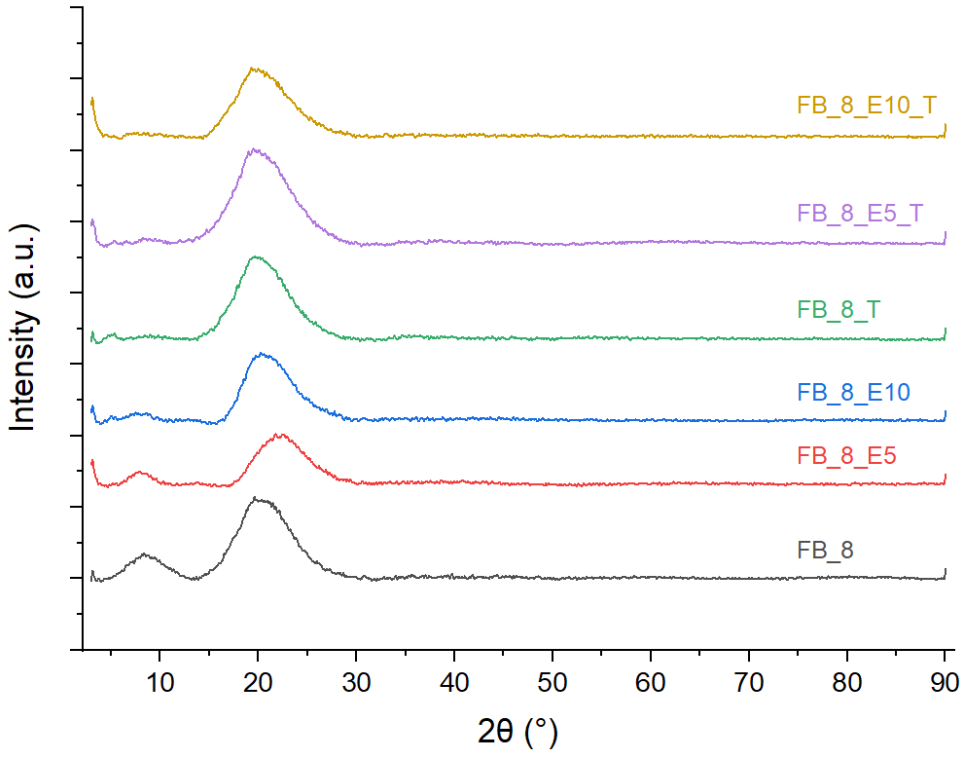


Figure 3.7. XRD patterns of the thyme oil added films

When thyme oil was incorporated, the first peak around 2θ = 8.94°, corresponding to α-helices, was significantly flattened, while the position and intensity of the second peak around 2θ = 19.58°, signifying β-sheets, remained mainly unchanged. This flattening of the first peak can be attributed to the non-crystalline nature of thyme oil. Similarly, in a previous study, the addition of marjoram essential oil flattened the first peak in the XRD spectra of mung bean isolate-pullulan-based films with high mung bean isolate ratios, due to the amorphous nature of the essential oil (Haghighatpanah et al., 2022). Likewise, the addition of rosemary essential oil reduced the peak intensities of starch-whey protein isolate-based films by forming an amorphous structure within the film matrix (Azevedo et al., 2019). This decrease and flattening were explained by the incorporation of non-crystalline essential oil droplets into the film network (Sani et al., 2021). A similar trend was observed in another study where thyme oil incorporation did not significantly alter the intensity of the second peak in soy protein isolate-based films, which was attributed to the good compatibility between the protein and the essential oil (Liu et al., 2024). MTGase addition at 5U dosage did not create a notable difference, but at 10U, the intensity of the second peak slightly decreased, due to the good compatibility between the enzyme and the protein (Zadeh et al., 2018). Thyme oil incorporation slightly increased the crystallinity index of the films, which ranged from 32.6% to 32.9% depending on the MTGase concentration. Similar findings were reported in a study on whey protein isolate-cellulose nanofiber composite films, where thyme oil addition increased the crystallinity index. This was attributed to a higher level of molecular interactions within the film network, which restricted the movement of polymer chains (Carvalho et al., 2020).

3.2.9 Antimicrobial activity of the films

Figure 3.13 shows the antimicrobial activity of the thyme oil-added films at pH 8 with varying dosages of MTGase. Results are shown in terms of inhibition zone.

Table 3.13. Antimicrobial activity of the thyme oil-added films

Film sample	*S. aureus* inhibition zone (mm)	*E.coli* inhibition zone (mm)
FB_8_T	10.00±0.00[a]	13.33±1.49[a]
FB_8_E5_T	10.00±0.00[a]	12.50±1.25[a]
FB_8_E10_T	10.00±0.00[a]	13.17±1.07[a]

*Different letters in the same column show significant differences ($p < 0.05$).

Thyme oil addition showed antimicrobial activity against *S. aureus* and *E. coli*. However, the MTGase concentration of the thyme oil-added films did not create any statistically significant variations in inhibition zone diameters. The inhibition zones created against *S. aureus* had the same diameter as the film discs, which were 10 mm. In contrast, thyme oil incorporation resulted in wider inhibition zones against *E. coli*, with diameters ranging from 12.50 to 13.33 mm depending on the MTGase concentration. Films prepared without thyme oil showed no antimicrobial activity against *S. aureus* or *E. coli*. These findings confirmed the results of a previous study, in which 1% thyme oil addition produced larger inhibition zones for *E. coli* compared to *S. aureus* (Emiroglu et al., 2010). Similarly, another study found that a 1% thyme oil addition into composite films resulted in lower cell viability for *E. coli* than *S. aureus* within the first 8 hours. However, the effects became similar between 8 and 24 hours (Sharma et al., 2020). In another study, it was reported that factors such as plant species and harvesting time can influence the antimicrobial activity of essential oils, as these variables affect the oil's composition, ultimately leading to variations in its antimicrobial properties (Kavas et al., 2015).

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

This study showed that faba bean protein concentrate can be used as a biodegradable packaging material. At pH values significantly higher than isoelectric point, faba bean protein had high solubility and could provide uniform films with good mechanical and barrier properties. pH change from 6 to 8 resulted in a reduction in WVP, thickness and antioxidant capacity, while increasing the opacity, mechanical properties, and the degree of cross-linking. In addition, microbial transglutaminase can successfully modify the physicochemical properties of protein-based films and can be used to alter their characteristics. The results showed that a higher degree of cross-linking was achieved with increasing MTGase concentrations. At pH 6, the addition of MTGase changed the mechanical and barrier properties of the films, but no significant differences were observed between 5U and 10U of MTGase. At pH 8, 5U of MTGase did not significantly affect the barrier properties, but reduced the EAB% and YM. However, these properties were recovered and slightly improved at 10U of MTGase. The best overall performance in mechanical and barrier properties was achieved with 10U of MTGase at pH 8, characterized by high moisture content, EAB%, and degree of cross-linking, as well as low water solubility and WVP. Lastly, thyme oil addition to protein-based biodegradable films can provide antimicrobial and antioxidant properties to the film, while modifying the physicochemical characteristics. The results showed that incorporating thyme oil increased the moisture content and thickness of the films, and created more opaque films with a darker color. At 10U of MTGase, thyme oil addition led to a reduction in the mechanical properties of the films. Furthermore, thyme oil incorporation significantly decreased the degree of cross-linking in the films. These films can be used to extend the shelf life of perishable foods like fresh produce, meat, seafood,

cheese, and bakery products, where moisture balance, oxidation prevention, and antimicrobial protection are crucial.

Different plasticizer concentrations can be studied in the future since the addition of the MTGase enzyme caused brittleness in the films. This may be overcome by incorporating more plasticizers in the system, which may help to achieve better films. In addition, processing conditions may be another potential subject, as the MTGase enzyme is very temperature-dependent, and different drying rates and temperatures may change the film characteristics in a better way.

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APPENDICES

A. Calibration Curves

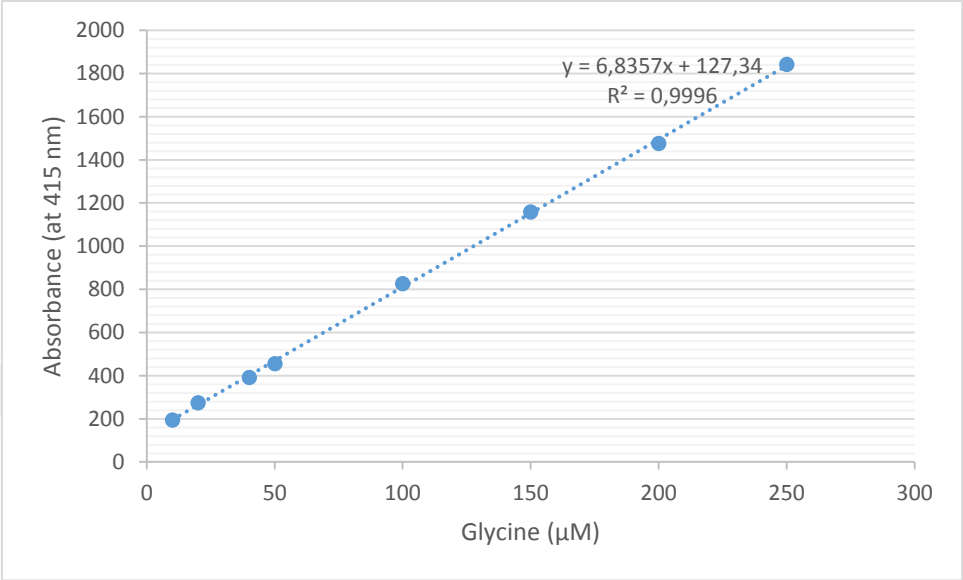


Figure A. 1. Calibration curve prepared with glycine for degree of crosslinking analysis

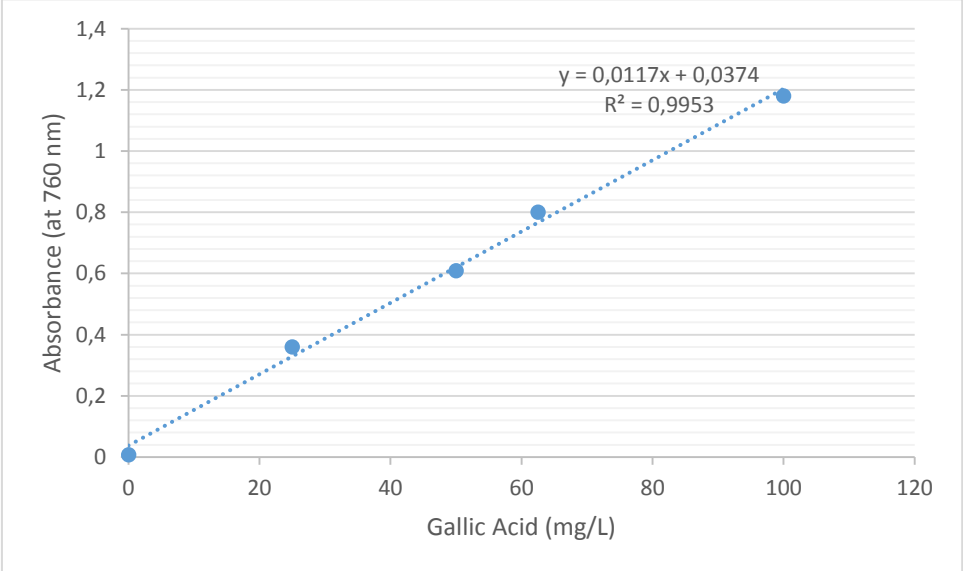


Figure A. 2. Calibration curve prepared with gallic acid for total phenolic content analysis

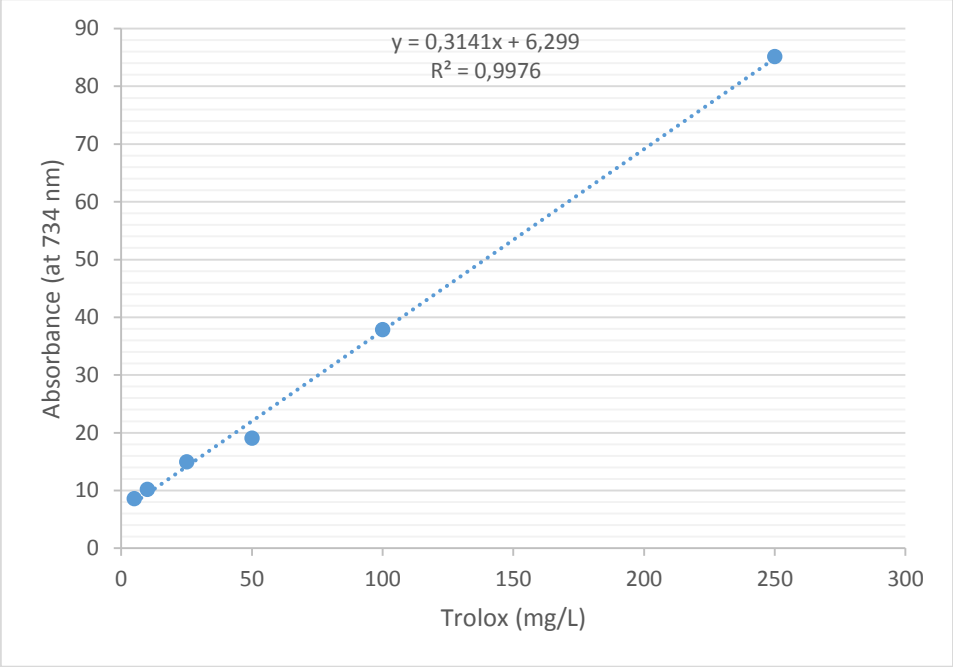


Figure A. 3. Calibration curve prepared with Trolox for total antioxidant activity analysis

B. Raw Material Graphs

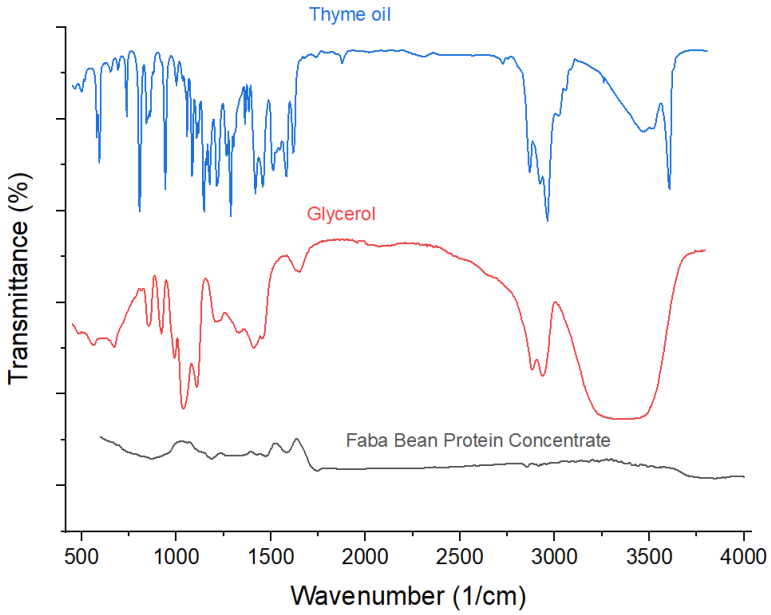


Figure B. 1. FTIR spectra of the raw materials (National Institute of Standards and Technology, n.d.; Catauro et al, 2017)

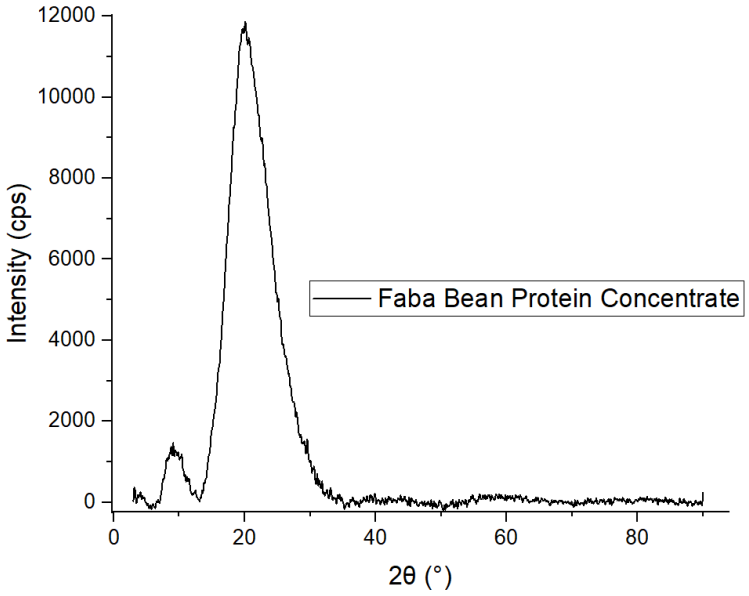


Figure B. 2. XRD pattern of the faba bean protein concentrate

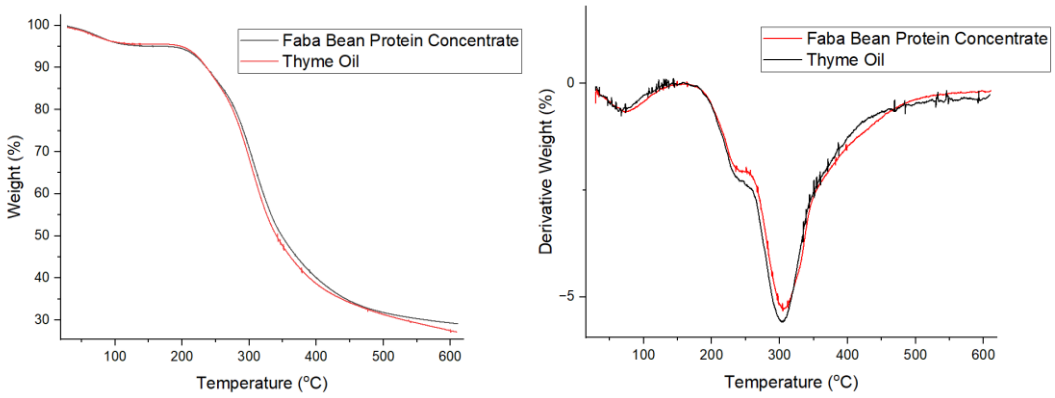


Figure B. 3. TGA thermograms and DTG curves of the faba bean protein concentrate and thyme oil

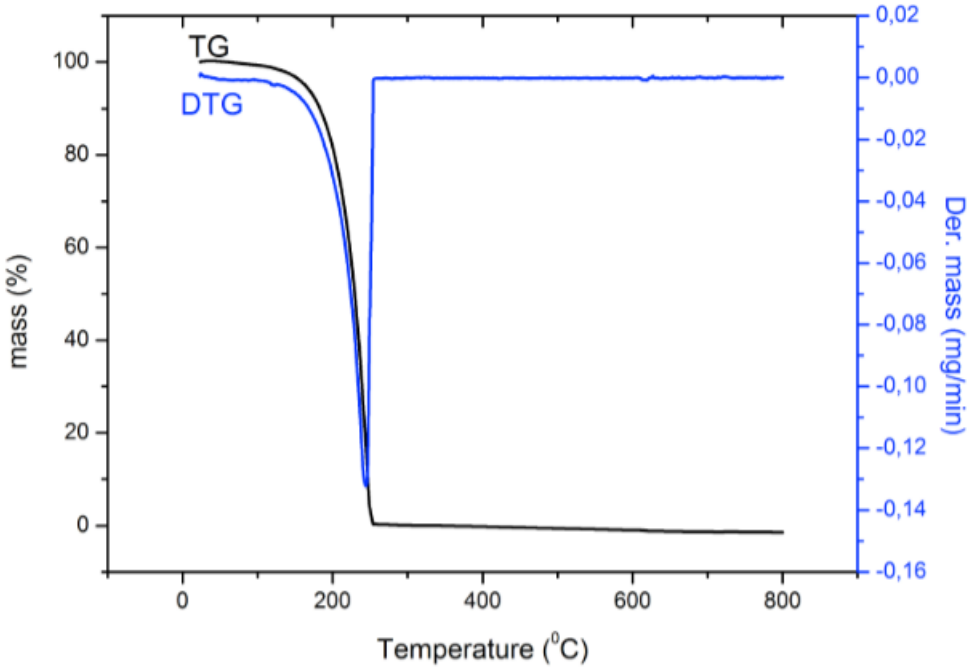


Figure B. 4. TGA thermogram and DTG curve of pure glycerol (Cordoba & Pagliuso, 2011)

C. Statistical Analysis

C.1. Effects of Different pH Values and MTGase Dosage

1. *Two-way ANOVA and Tukey's comparison test for Thickness (mm) versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	0,003540	0,003540	34,14	0,000
Enzyme Dosage	2	0,000422	0,000211	2,03	0,149
pH*Enzyme Dosage	2	0,000632	0,000316	3,05	0,062
Error	30	0,003111	0,000104		
Total	35	0,007704			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,0101825	59,63%	52,90%	41,86%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 0	6	0,183667	A		
6 5	6	0,174167	A	B	
6 10	6	0,165833	A	B	C
8 10	6	0,157333		B	C
8 0	6	0,155167			C
8 5	6	0,151667			C

Means that do not share a letter are significantly different.

2. *Two-way ANOVA and Tukey's comparison test for %Moisture versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	7,816	7,816	5,80	0,022
Enzyme Dosage	2	69,465	34,733	25,77	0,000
pH*Enzyme Dosage	2	4,689	2,344	1,74	0,193
Error	30	40,435	1,348		
Total	35	122,404			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1,16096	66,97%	61,46%	52,43%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 10	6	16,2610	A		
6 5	6	16,1359	A		
8 10	6	15,4149	A		
8 5	6	14,2803	A		B
6 0	6	12,6742			B
8 0	6	12,5803			B

Means that do not share a letter are significantly different.

3. *Two-way ANOVA and Tukey's comparison test for %Solubility versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	20,301	20,301	4,99	0,033
Enzyme Dosage	2	26,450	13,225	3,25	0,053
pH*Enzyme Dosage	2	3,789	1,895	0,47	0,632
Error	30	122,034	4,068		
Total	35	172,574			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,01688	29,29%	17,50%	0,00%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping
8 0	6	35,2602	A
8 5	6	35,0362	A
6 0	6	34,5306	A
8 10	6	33,5255	A
6 5	6	32,7189	A
6 10	6	32,0668	A

Means that do not share a letter are significantly different.

4. *Two-way ANOVA and Tukey's comparison test for WVP versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	0,43825	0,43825	21,09	0,001
Enzyme Dosage	2	0,09746	0,04873	2,34	0,138
pH*Enzyme Dosage	2	0,10251	0,05126	2,47	0,127
Error	12	0,24937	0,02078		
Total	17	0,88759			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,144154	71,91%	60,20%	36,79%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 0	3	2,60328	A		
6 5	3	2,49650	A	B	
6 10	3	2,32666	A	B	C
8 5	3	2,29772	A	B	C
8 10	3	2,11461		B	C
8 0	3	2,07789			C

Means that do not share a letter are significantly different.

5. *Two-way ANOVA and Tukey's comparison test for L* versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	1268,15	1268,15	212,69	0,000
Enzyme Dosage	2	73,17	36,59	6,14	0,004
pH*Enzyme Dosage	2	37,65	18,82	3,16	0,049
Error	66	393,53	5,96		
Total	71	1772,50			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,44183	77,80%	76,12%	73,58%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 10	12	95,7633	A		
6 5	12	95,1625	A		
6 0	12	95,1550	A		
8 10	12	89,4150		B	
8 0	12	85,7475			C
8 5	12	85,7375			C

Means that do not share a letter are significantly different.

6. *Two-way ANOVA and Tukey's comparison test for a* versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	15,633	15,6334	113,32	0,000
Enzyme Dosage	2	1,502	0,7512	5,45	0,006
pH*Enzyme Dosage	2	1,048	0,5238	3,80	0,027
Error	66	9,106	0,1380		
Total	71	27,289			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,371433	66,63%	64,11%	60,29%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 0	12	-1,78000	A		
8 5	12	-2,11667	A	B	
8 10	12	-2,42917		B	
6 0	12	-3,01083			C
6 5	12	-3,04167			C
6 10	12	-3,06917			C

Means that do not share a letter are significantly different.

7. *Two-way ANOVA and Tukey's comparison test for b* versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	73,61	73,609	16,06	0,000
Enzyme Dosage	2	10,07	5,035	1,10	0,339
pH*Enzyme Dosage	2	64,95	32,474	7,09	0,002
Error	66	302,43	4,582		
Total	71	451,06			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,14063	32,95%	27,87%	20,21%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 0	12	19,1908	A		
8 5	12	17,9358	A	B	
6 5	12	16,8783	A	B	C
8 10	12	16,6650	A	B	C
6 10	12	16,3317		B	C
6 0	12	14,5150			C

Means that do not share a letter are significantly different.

8. *Two-way ANOVA and Tukey's comparison test for ΔE* versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	454,82	454,825	81,36	0,000
Enzyme Dosage	2	43,96	21,981	3,93	0,024
pH*Enzyme Dosage	2	95,98	47,991	8,58	0,000
Error	66	368,98	5,591		
Total	71	963,75			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,36445	61,71%	58,81%	54,44%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 0	12	21,4118	A		
8 5	12	20,6218	A		
8 10	12	17,4322		B	
6 5	12	15,7578		B	C
6 10	12	15,1488		B	C
6 0	12	13,4790			C

Means that do not share a letter are significantly different.

9. *Two-way ANOVA and Tukey's comparison test for Opacity versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	7,3391	7,33910	194,62	0,000
Enzyme Dosage	2	0,3694	0,18472	4,90	0,010
pH*Enzyme Dosage	2	0,2603	0,13016	3,45	0,038
Error	66	2,4888	0,03771		
Total	71	10,4577			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,194190	76,20%	74,40%	71,68%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 5	12	0,946002	A		
8 0	12	0,909951	A		
8 10	12	0,650375		B	
6 0	12	0,209107			C
6 5	12	0,201577			C
6 10	12	0,180038			C

Means that do not share a letter are significantly different.

10. *Two-way ANOVA and Tukey's comparison test for WI versus pH; Enzyme Dosage*

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	567,48	567,484	98,43	0,000
Enzyme Dosage	2	48,18	24,088	4,18	0,020
pH*Enzyme Dosage	2	95,76	47,881	8,31	0,001
Error	66	380,50	5,765		
Total	71	1091,92			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,40107	65,15%	62,51%	58,53%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 0	12	84,3843	A		
6 10	12	82,8422	A	B	
6 5	12	82,1730	A	B	
8 10	12	79,9794		B	
8 5	12	76,6986			C
8 0	12	75,8769			C

Means that do not share a letter are significantly different.

11. Two-way ANOVA and Tukey's comparison test for YI versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
pH	Fixed	2	6; 8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	60,31	30,16	2,36	0,103
pH	1	585,92	585,92	45,79	0,000
Enzyme Dosage*pH	2	193,50	96,75	7,56	0,001
Error	66	844,60	12,80		
Total	71	1684,34			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3,57729	49,86%	46,06%	40,32%

Tukey Pairwise Comparisons: Enzyme Dosage*pH

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*pH	N	Mean	Grouping			
0 8	12	31,9178	A			
5 8	12	30,0922	A	B		
10 8	12	26,6290		B	C	
5 6	12	25,3520			C	D
10 6	12	24,3686			C	D
0 6	12	21,8023				D

Means that do not share a letter are significantly different.

12. Two-way ANOVA and Tukey's comparison test for TS versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	40,4390	40,4390	57,74	0,000
Enzyme Dosage	2	14,3911	7,1955	10,27	0,000
pH*Enzyme Dosage	2	0,0784	0,0392	0,06	0,946
Error	30	21,0103	0,7003		
Total	35	75,9188			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,836865	72,33%	67,71%	60,15%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 0	6	7,03144	A		
8 10	6	5,83123	A	B	
8 5	6	5,72749	A	B	
6 0	6	5,01986		B	C
6 10	6	3,72300			C D
6 5	6	3,48813			D

Means that do not share a letter are significantly different.

13. Two-way ANOVA and Tukey's comparison test for %EAB versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	1995,0	1995,00	107,11	0,000
Enzyme Dosage	2	146,8	73,42	3,94	0,030
pH*Enzyme Dosage	2	124,4	62,18	3,34	0,049
Error	30	558,8	18,63		
Total	35	2825,0			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4,31579	80,22%	76,92%	71,52%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 10	6	23,9125	A		
8 0	6	22,4375	A	B	
8 5	6	16,3175		B	C
6 0	6	8,8288			C D
6 5	6	5,2047			D
6 10	6	3,9685			D

Means that do not share a letter are significantly different.

14. Two-way ANOVA and Tukey's comparison test for YM versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	51594	51593,6	32,14	0,000
Enzyme Dosage	2	8526	4263,1	2,66	0,087
pH*Enzyme Dosage	2	1413	706,6	0,44	0,648
Error	30	48165	1605,5		
Total	35	109698			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
40,0686	56,09%	48,78%	36,77%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
8 0	6	249,146	A		
8 10	6	246,542	A		
8 5	6	227,568	A	B	
6 0	6	189,576	A	B	C
6 10	6	169,085		B	C
6 5	6	137,453			C

Means that do not share a letter are significantly different.

15. Two-way ANOVA and Tukey's comparison test for Cross-linking(Glycine/g) versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	572,7	572,7	2,60	0,118
Enzyme Dosage	2	2014,3	1007,1	4,57	0,019
pH*Enzyme Dosage	2	217,4	108,7	0,49	0,616
Error	30	6617,0	220,6		
Total	35	9421,2			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
14,8514	29,77%	18,06%	0,00%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping	
6 0	6	86,4081	A	
8 0	6	83,0800	A	B
6 5	6	76,0702	A	B
6 10	6	74,0465	A	B
8 5	6	70,2430	A	B
8 10	6	59,2712		B

Means that do not share a letter are significantly different.

16. Two-way ANOVA and Tukey's comparison test for TPC(GAE/g) versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	5,5147	5,5147	44,74	0,000
Enzyme Dosage	2	2,6396	1,3198	10,71	0,000
pH*Enzyme Dosage	2	0,4961	0,2481	2,01	0,151
Error	30	3,6977	0,1233		
Total	35	12,3482			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,351081	70,05%	65,06%	56,88%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 10	6	3,64687	A		
6 5	6	3,32564	A	B	
6 0	6	2,72867		B	C
8 5	6	2,58707			C
8 10	6	2,55698			C
8 0	6	2,20878			C

Means that do not share a letter are significantly different.

17. Two-way ANOVA and Tukey's comparison test for ABTS(TE/g) versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	164593	164593	32,59	0,000
Enzyme Dosage	2	58	29	0,01	0,994
pH*Enzyme Dosage	2	26864	13432	2,66	0,086
Error	30	151528	5051		
Total	35	343043			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
71,0699	55,83%	48,47%	36,39%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 10	6	763,500	A		
6 0	6	755,078	A		
6 5	6	700,792	A	B	
8 5	6	642,569	A	B	
8 0	6	586,765		B	
8 10	6	584,336		B	

Means that do not share a letter are significantly different.

18. Two-way ANOVA and Tukey's comparison test for %DPPH Inhibition versus pH; Enzyme Dosage

Factor Information

Factor	Type	Levels	Values
pH	Fixed	2	6; 8
Enzyme Dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	1	5,97009	5,97009	437,58	0,000
Enzyme Dosage	2	0,21436	0,10718	7,86	0,002
pH*Enzyme Dosage	2	0,09333	0,04666	3,42	0,046
Error	30	0,40930	0,01364		
Total	35	6,68708			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,116805	93,88%	92,86%	91,19%

Tukey Pairwise Comparisons: pH*Enzyme Dosage

Grouping Information Using the Tukey Method and 95% Confidence

pH*Enzyme Dosage	N	Mean	Grouping		
6 10	6	1,32178	A		
6 5	6	1,28585	A		
6 0	6	1,03400		B	
8 5	6	0,41842			C
8 10	6	0,41784			C
8 0	6	0,36200			C

Means that do not share a letter are significantly different.

C.2. Effects of Different MTGase Dosage and Thyme Oil Addition

1. *Two-way ANOVA and Tukey's comparison test for Thickness (mm) versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	0,000050	0,000025	0,49	0,618
Thyme Oil	1	0,001457	0,001457	28,43	0,000
Enzyme Dosage*Thyme Oil	2	0,000159	0,000079	1,55	0,229
Error	30	0,001537	0,000051		
Total	35	0,003203			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,0071581	52,00%	44,01%	30,89%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
0 1	6	0,170167	A		
5 1	6	0,168000	A	B	
10 1	6	0,164167	A	B	C
10 0	6	0,157333		B	C
0 0	6	0,155167			C
5 0	6	0,151667			C

Means that do not share a letter are significantly different

2. *Two-way ANOVA and Tukey's comparison test for %Moisture versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	31,618	15,809	11,17	0,000
Thyme Oil	1	14,601	14,601	10,32	0,003
Enzyme Dosage*Thyme Oil	2	2,317	1,158	0,82	0,451
Error	30	42,444	1,415		
Total	35	90,980			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1,18945	53,35%	45,57%	32,82%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
10 1	6	15,9904	A		
5 1	6	15,7600	A		
10 0	6	15,4149	A		
0 1	6	14,3462	A	B	
5 0	6	14,2803	A	B	
0 0	6	12,5803		B	

Means that do not share a letter are significantly different.

3. *Two-way ANOVA and Tukey's comparison test for %Solubility versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	29,579	14,790	6,69	0,004
Thyme Oil	1	52,443	52,443	23,73	0,000
Enzyme Dosage*Thyme Oil	2	5,505	2,752	1,25	0,302
Error	30	66,287	2,210		
Total	35	153,813			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1,48646	56,90%	49,72%	37,94%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
0 1	6	38,6353	A		
5 1	6	36,4956	A	B	
10 1	6	35,9326		B	C
0 0	6	35,2602		B	C
5 0	6	35,0362		B	C
10 0	6	33,5255			C

Means that do not share a letter are significantly different.

4. *Two-way ANOVA and Tukey's comparison test for WVP versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	0,03260	0,01630	0,94	0,419
Thyme Oil	1	0,06033	0,06033	3,46	0,087
Enzyme Dosage*Thyme Oil	2	0,06416	0,03208	1,84	0,201
Error	12	0,20896	0,01741		
Total	17	0,36605			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,131961	42,91%	19,13%	0,00%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping
10 1	3	2,33367	A
5 0	3	2,29772	A
0 1	3	2,25773	A
5 1	3	2,24617	A
10 0	3	2,11461	A
0 0	3	2,07789	A

Means that do not share a letter are significantly different.

5. *Two-way ANOVA and Tukey's comparison test for L* versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	2,05	1,03	0,11	0,896
Thyme Oil	1	1445,16	1445,16	155,64	0,000
Enzyme Dosage*Thyme Oil	2	227,25	113,63	12,24	0,000
Error	66	612,84	9,29		
Total	71	2287,31			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3,04721	73,21%	71,18%	68,11%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
10 0	12	89,4150	A		
0 0	12	85,7475		B	
5 0	12	85,7375		B	
5 1	12	79,6917			C
0 1	12	78,8750			C D
10 1	12	75,4525			D

Means that do not share a letter are significantly different.

6. *Two-way ANOVA and Tukey's comparison test for a* versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	0,1721	0,0861	0,75	0,475
Thyme Oil	1	16,9653	16,9653	148,55	0,000
Enzyme Dosage*Thyme Oil	2	4,4289	2,2145	19,39	0,000
Error	66	7,5378	0,1142		
Total	71	29,1042			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,337949	74,10%	72,14%	69,18%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping
10 1	12	-0,80333	A
5 1	12	-1,25583	B
0 1	12	-1,35417	B
0 0	12	-1,78000	C
5 0	12	-2,11667	C D
10 0	12	-2,42917	D

Means that do not share a letter are significantly different.

7. *Two-way ANOVA and Tukey's comparison test for b* versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	13,94	6,972	1,62	0,205
Thyme Oil	1	367,25	367,250	85,40	0,000
Enzyme Dosage*Thyme Oil	2	90,97	45,484	10,58	0,000
Error	66	283,82	4,300		
Total	71	755,98			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,07371	62,46%	59,61%	55,32%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping
10 1	12	24,3392	A
0 1	12	21,8058	B
5 1	12	21,1975	B C
0 0	12	19,1908	C D
5 0	12	17,9358	D E
10 0	12	16,6650	E

Means that do not share a letter are significantly different.

8. *Two-way ANOVA and Tukey's comparison test for ΔE* versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	14,49	7,24	0,87	0,422
Thyme Oil	1	1391,73	1391,73	168,02	0,000
Enzyme Dosage*Thyme Oil	2	272,63	136,31	16,46	0,000
Error	66	546,68	8,28		
Total	71	2225,53			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,87803	75,44%	73,58%	70,77%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
10 1	12	31,7276	A		
0 1	12	27,5668		B	
5 1	12	26,5507		B	
0 0	12	21,4118			C
5 0	12	20,6218			C D
10 0	12	17,4322			D

Means that do not share a letter are significantly different.

9. *Two-way ANOVA and Tukey's comparison test for Opacity versus Enzyme Dosage; Thyme Oil*

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	0,0185	0,00924	0,17	0,841
Thyme Oil	1	5,9308	5,93078	111,26	0,000
Enzyme Dosage*Thyme Oil	2	1,3699	0,68493	12,85	0,000
Error	66	3,5183	0,05331		
Total	71	10,8374			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,230883	67,54%	65,08%	61,37%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
10 1	12	1,61452	A		
5 1	12	1,32411		B	
0 1	12	1,28973		B	
5 0	12	0,94600			C
0 0	12	0,90995			C D
10 0	12	0,65037			D

Means that do not share a letter are significantly different.

10. Two-way ANOVA and Tukey's comparison test for WI versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	14,12	7,06	0,83	0,441
Thyme Oil	1	1452,40	1452,40	170,46	0,000
Enzyme Dosage*Thyme Oil	2	280,88	140,44	16,48	0,000
Error	66	562,36	8,52		
Total	71	2309,76			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,91901	75,65%	73,81%	71,02%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping
10 0	12	79,9794	A
5 0	12	76,6986	A B
0 0	12	75,8769	B
5 1	12	70,6073	C
0 1	12	69,5882	C
10 1	12	65,4113	D

Means that do not share a letter are significantly different.

11. Two-way ANOVA and Tukey's comparison test for YI versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	66,89	33,45	1,77	0,178
Thyme Oil	1	2499,17	2499,17	132,49	0,000
Enzyme Dosage*Thyme Oil	2	550,07	275,04	14,58	0,000
Error	66	1244,97	18,86		
Total	71	4361,10			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4,34318	71,45%	69,29%	66,03%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping
10 1	12	46,2265	A
0 1	12	39,5915	B
5 1	12	38,1705	B
0 0	12	31,9178	C
5 0	12	30,0922	C D
10 0	12	26,6290	D

Means that do not share a letter are significantly different.

12. Two-way ANOVA and Tukey's comparison test for TS versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	11,798	5,8989	11,47	0,000
Thyme Oil	1	11,127	11,1274	21,64	0,000
Enzyme Dosage*Thyme Oil	2	1,295	0,6476	1,26	0,298
Error	30	15,423	0,5141		
Total	35	39,644			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,717017	61,10%	54,61%	43,98%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
0 0	6	7,03144	A		
10 0	6	5,83123	A	B	
0 1	6	5,81731	A	B	
5 0	6	5,72749		B	
5 1	6	5,12276		B	C
10 1	6	4,31432			C

Means that do not share a letter are significantly different.

13. Two-way ANOVA and Tukey's comparison test for %EAB versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	167,62	83,81	3,09	0,060
Thyme Oil	1	82,57	82,57	3,05	0,091
Enzyme Dosage*Thyme Oil	2	247,64	123,82	4,57	0,019
Error	30	813,09	27,10		
Total	35	1310,91			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
5,20605	37,98%	27,64%	10,68%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping	
10 0	6	23,9125	A	
0 0	6	22,4375	A	B
0 1	6	22,1229	A	B
5 1	6	17,9104	A	B
5 0	6	16,3175	A	B
10 1	6	13,5476		B

Means that do not share a letter are significantly different.

14. Two-way ANOVA and Tukey's comparison test for YM versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	2706	1353	1,10	0,345
Thyme Oil	1	20193	20193	16,44	0,000
Enzyme Dosage*Thyme Oil	2	4179	2089	1,70	0,200
Error	30	36839	1228		
Total	35	63917			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
35,0421	42,36%	32,76%	17,00%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
0 0	6	249,146	A		
10 0	6	246,542	A		
5 0	6	227,568	A	B	
0 1	6	208,933	A	B	
5 1	6	202,277	A	B	
10 1	6	169,943		B	

Means that do not share a letter are significantly different.

15. Two-way ANOVA and Tukey's comparison test for Cross-linking (Glycine/g) versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	1680,6	840,31	9,36	0,001
Thyme Oil	1	1601,8	1601,82	17,84	0,000
Enzyme Dosage*Thyme Oil	2	302,2	151,10	1,68	0,203
Error	30	2692,9	89,76		
Total	35	6277,6			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
9,47439	57,10%	49,95%	38,23%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
0 1	6	89,1389	A		
5 1	6	83,9699	A	B	
0 0	6	83,0800	A	B	
10 1	6	79,5081	A	B	
5 0	6	70,2430		B	C
10 0	6	59,2712			C

Means that do not share a letter are significantly different.

16. Two-way ANOVA and Tukey's comparison test for TPC(GAE/g) versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	4,46	2,23	3,11	0,059
Thyme Oil	1	1187,35	1187,35	1654,71	0,000
Enzyme Dosage*Thyme Oil	2	3,21	1,61	2,24	0,124
Error	30	21,53	0,72		
Total	35	1216,55			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,847089	98,23%	97,94%	97,45%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping		
5 1	6	14,7954	A		
0 1	6	13,7128	A	B	
10 1	6	13,3026		B	
5 0	6	2,5871			C
10 0	6	2,5570			C
0 0	6	2,2088			C

Means that do not share a letter are significantly different.

17. Two-way ANOVA and Tukey's comparison test for ABTS(TE/g) versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	10629	5315	1,95	0,160
Thyme Oil	1	1284816	1284816	471,38	0,000
Enzyme Dosage*Thyme Oil	2	3494	1747	0,64	0,534
Error	30	81769	2726		
Total	35	1380708			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
52,2076	94,08%	93,09%	91,47%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping	
5 1	6	992,667	A	
0 1	6	980,792	A	
10 1	6	973,708	A	
5 0	6	642,569		B
0 0	6	586,765		B
10 0	6	584,336		B

Means that do not share a letter are significantly different.

18. Two-way ANOVA and Tukey's comparison test for %DPPH Inhibition versus Enzyme Dosage; Thyme Oil

Factor Information

Factor	Type	Levels	Values
Enzyme Dosage	Fixed	3	0; 5; 10
Thyme Oil	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme Dosage	2	0,016732	0,008366	2,26	0,121
Thyme Oil	1	0,808403	0,808403	218,86	0,000
Enzyme Dosage*Thyme Oil	2	0,001710	0,000855	0,23	0,795
Error	30	0,110813	0,003694		
Total	35	0,937658			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,0607762	88,18%	86,21%	82,98%

Tukey Pairwise Comparisons: Enzyme Dosage*Thyme Oil

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme Dosage*Thyme Oil	N	Mean	Grouping
5 1	6	0,721046	A
10 1	6	0,699390	A
0 1	6	0,676933	A
5 0	6	0,418421	B
10 0	6	0,417838	B
0 0	6	0,361998	B

Means that do not share a letter are significantly different.

19. Two-way ANOVA and Tukey's comparison test for Inhibition Zone (mm) versus Enzyme dosage

Factor Information

Factor	Type	Levels	Values
Enzyme dosage	Fixed	3	0; 5; 10

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Enzyme dosage	2	2,333	1,167	0,59	0,567
Error	15	29,667	1,978		
Total	17	32,000			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1,40633	7,29%	0,00%	0,00%

Tukey Pairwise Comparisons: Enzyme dosage

Grouping Information Using the Tukey Method and 95% Confidence

Enzyme dosage	N	Mean	Grouping
0	6	13,3333	A
10	6	13,1667	A
5	6	12,5000	A

Means that do not share a letter are significantly different.